KU Leuven
Group Biomedical Sciences
Department of Pharmaceutical and Pharmacological Sciences
Laboratory for Therapeutic and Diagnostic antibodies

Evaluation of TAFI Zymogen Activity

and

Various TAFI Activators in Regulation of Fibrinolysis

Niraj MISHRA

Doctoral thesis in Pharmaceutical Sciences

Leuven, 16.09.2013

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It always seems impossible until it is done : Nelson Mandela

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Jury:

Promoter: Prof. Dr. Ann Gils
Co-Promoter: Prof. Dr. Paul Declerck
Chair: Prof. Dr. Myriam Baes
Secretary: Prof. Dr. Sergei.Strelkov
Jury members: Prof. Dr. Mario Colucci
Prof. Dr. Ingrid De Meester
Dr. Ilse Scroyen

Doctoral thesis in Pharmaceutical Sciences

Leuven, 16.09.2013

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Auditorium Kasteel van Arenberg

Kasteelpark Arenberg I

3001 Heverlee

Promoter: Prof. Ann Gils
Co-Promoter: Prof. Paul Declerck

Laboratory for Therapeutic and Diagnostic antibodies
Department of Pharmaceutical and Pharmacological Sciences
Herestraat 49, O&NII, PB 820
3000 Leuven
Belgium

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This is the most significant and toughest section of my thesis. Almost four years ago I started my Ph.D. in the Laboratory for Therapeutic and Diagnostic Antibodies, Department of Pharmaceutical and Pharmacological Sciences at KU Leuven, Leuven, Belgium. New lab, new country, new culture, new challenges and a lot to be learnt!! Although there were some anxious moments, but now when I look back, it seems like a sojourn full of pleasant and successful happenings encompassing few of the most unforgettable days of my life. Events of last four years provided me with an amazing opportunity to learn a lot on different fronts of my life - professional as well as personal. Most importantly, I understood that patience is the key to success which kept me motivated even during challenging phases of my Ph.D. Great foundations are not laid overnight and I hope my doctorate is a little step towards my dreams. Pharmaceutical biologists frequently assess their achievements in terms of the number of innovative drugs they discovered, the novel interactions and working mechanism of these drugs and very often in terms of the number of high impact publications. But I constantly believed that a researcher's endeavours are always measured in terms of the number of professional and personal relationships one has secured in one's career. It would have been impossible to complete my Ph.D. without the assistance of people surrounding me both in Belgium and back home in India. I am writing this acknowledgement section with immense sense of gratification and appreciation to all those who helped me achieve this feat (Ph.D.) in my professional career.

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-Niraj

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Table of Contents

List of Abbreviations

α_2-AP	α_2-antiplasmin
Abs	Antibodies
BSA	Bovine Serum Albumin
CDR	Complementarity Determining Region
CLT	50% Clot-Lysis Time
CPN	Carboxypeptidase N
CVDs	CardioVascular Diseases
E. coli	*Escherichia coli*
EC_{50}	Half maximal effective concentration
ELISA	Enzyme-Linked ImmunoSorbent Assay
FDP	Fibrin Degradation Product
GEMSA	GuanidinoEthyl-MercaptoSuccinic Acid
HCAbs	Heavy-chain antibodies
HEK293T	Human Embryonic Kidney cells containing SV40 large T-antigen
HRP	HorseRadish Peroxidase
hTAFI mice	TAFI single-gene-deficient mice expressing human TAFI
Igs	Immunoglobulins
iv	Intravenous
K_A	Affinity constants
k_a	association rate
k_{cat}	catalytic rate
k_{cat}/K_M	catalytic efficiency
kDa	kiloDalton
K_M	Michaelis-Menten constant
L_{60}	Percentage lysis 60 min from start of clot formation
LPS	LipoPolySaccharide
MA	Monoclonal Antibody/Antibodies
MI	Myocardial Infarction

ML	Maximum Lysis
Nb	Nanobody
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PPACK	H-D-Phenylalanyl-L-Prolyl-L-Arginine Chloromethyl Ketone
PTCI	Potato Tuber Carboxypeptidase Inhibitor
RAM	Rabbit Anti-Mouse
Rotem	ROtational ThromboElastoMetry
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SEM	Standard Error of Mean
SPR	Surface Plasmon Resonance
T/TM	Thrombin/ThromboModulin
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TAFI(a)	TAFI/activated TAFI
TAFI KO	TAFI single-gene-deficient mice
TAFIa	Activated Thrombin Activatable Fibrinolysis Inhibitor
TAFI-ACIIYQ	TAFI-A147-S305C-T325I-T329I-H333Y-H335Q
TAFIai	Conformationally inactivated TAFIa
TAFI-TI	Natural TAFI variant TAFI-T^{147}-I^{325}
TBS	Tris-Buffered Saline
TDP	TAFI-Depleted Plasma
TF	Tissue-Factor
t-PA	tissue-type Plasminogen Activator
u-PA	urokinase-type Plasminogen Activator
vWF	von Willebrand factor
WT	Wild-Type

List of Mainly Used Monoclonal Antibodies and Nanobodies

MA-TCK11A9 (Mishra, *et al.*, Thromb Haemost, 2011)	• Selectively inhibits the plasmin-mediated human TAFI activation • Major residues involved: Lys^{268}, Ser^{272} and Arg^{276}
MA-TCK22G2 (Mishra, *et al.*, Thromb Haemost, 2011)	• Selectively inhibits the plasmin- and thrombin-mediated human TAFI activation • Major residues involved: Thr^{147} and Ala^{148}
MA-TCK27A4 (Mishra, *et al.*, Thromb Haemost, 2011)	• Inhibits thrombin-, thrombin/thrombomodulin (T/TM)- and plasmin-mediated human TAFI activation • Major residues involved: Phe^{113}
MA-TCK26D6 (Vercauteren *et al.*, Blood, 2011)	• Mainly inhibits plasmin-mediated human, mouse and rat TAFI activation • Major residues involved: Asp^{87} and Thr^{88}
MA-T12D11 (Gils *et al.*, J Thromb Haemost, 2004)	• Selectively inhibits T/TM-mediated human TAFI activation • Major residues involved: Gly^{66}
MA-Tom1-41B2 (Vandevenne *et al.*, J Immunol Methods, 2009)	• Control antibody directed toward tomato pectin methylesterase
Vhh-TAFI-a51 (Mishra *et al.*, J Thromb Haemost, 2012)	• Stimulates zymogen activity of human TAFI • Major residues involved: Asp^{75} and Thr^{301}
Vhh-TAFI-i103 (Mishra *et al.*, J Thromb Haemost, 2012)	• Stimulates zymogen activity of human TAFI • Major residues involved: Asp^{75} and Thr^{301}

CHAPTER 1

General Introduction

1.1. Hemostasis and Thrombosis

The hemostatic process maintains the vascular integrity of our body and needs to be immediately activated after tissue injury to reduce the extravasation of blood from the vasculature. Hemostasis represents normal physiological condition. However, strict regulation in thrombus formation at site of injury is important to maintain the circulation. Therefore, hemostasis represents a delicate balance between coagulation (thrombus formation) and fibrinolysis (thrombus dissolution). Slight disturbance of this natural dynamic balance due to genetic (haemophilia, mutations in von Willebrand factor (*vWF*) and factor V and deficiency of prothrombin, protein C, protein S and antithrombin, etc.) or acquired (pregnancy, surgery, obesity, hormonal therapy, anticoagulant therapy, autoimmune disorders, and cancer, etc.) factors could lead to either thrombotic or hemorrhagic complications (Fig. 1.1) [1-5].

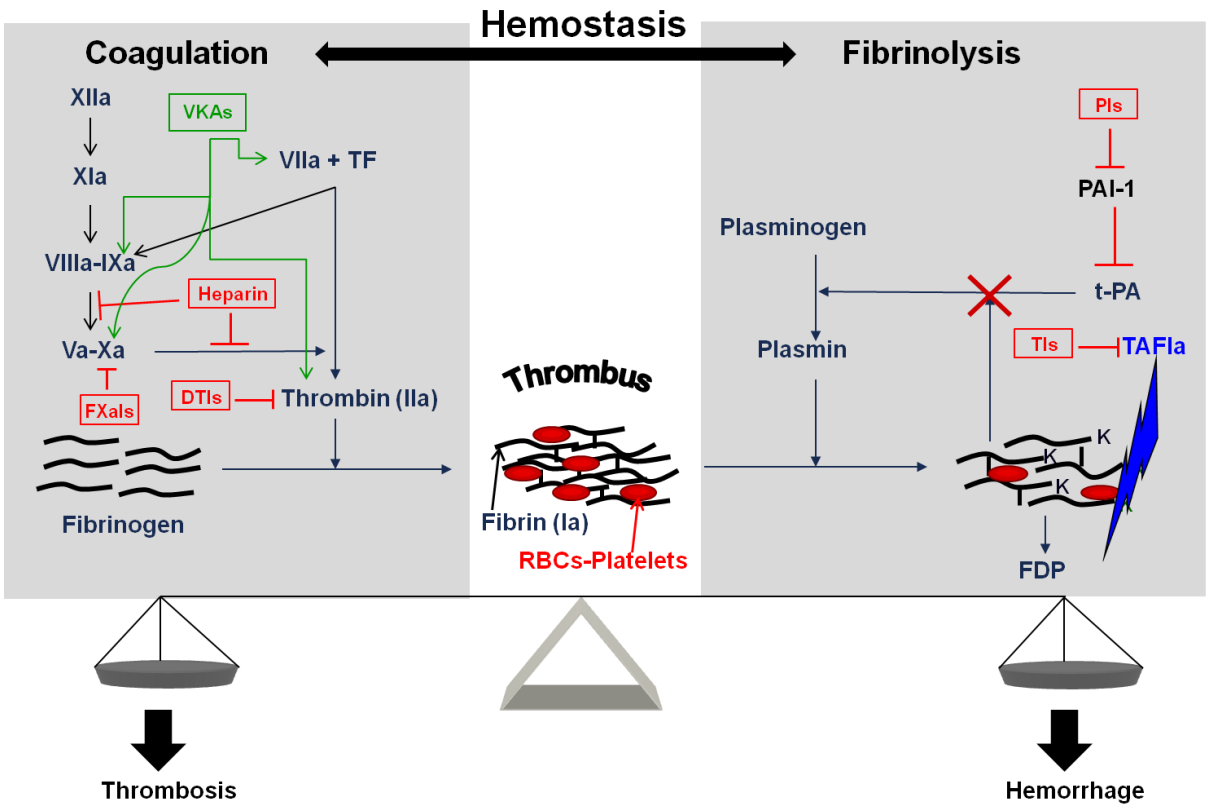

Figure 1.1: Regulation of hemostatic system (adapted from Mishra *et al.* [6]). Exposure of tissue factor (TF) with blood and some pathological conditions (atherosclerosis and bacterial infection) trigger activation of the coagulation cascade through the activation of extrinsic pathway (factor IX and factor X) or intrinsic pathway (factor XIa and factor XIIa), respectively which results in formation of first hemostatic plug and ultimately thrombus. During fibrinolysis, t-PA- and/or u-PA-mediated activation of plasminogen degrades fibrin blood clot into fibrin degradation product (FDP). Plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin (α_2-AP) and activated thrombin-activatable fibrinolysis inhibitor (TAFIa) work as inhibitory molecules in fibrinolysis. Targets of anticoagulants [vitamin K antagonists (VKAs), heparins, direct thrombin inhibitors (DTIs) and factor Xa inhibitors (FXaIs)] and thrombolytic drugs [t-PA, PAI-1 inhibitors (PIs) and TAFI inhibitors (TIs)] are also indicated. RBCs; red blood cells.

1.1.1. The coagulation system

The coagulation system is a network of sequentially interacting coagulation factors which ultimately leads to the formation of a stable blood clot/thrombus. The current “cell-based three phase coagulation model” of the coagulation highlights (a) tissue factor (TF) as the major regulator of thrombus formation

(initiation phase), (b) the necessity of the rapid amplification of thrombin generation for development of stable clot (amplification phase) and (c) the simultaneous involvement of cellular elements (activated platelets) and coagulation factors in stabilization of a hemostatic plug (propagation phase) [7]. A hemostatic plug is formed due to the earliest response to vessel wall injury and involves *vWF*- and fibrinogen-mediated anchoring of platelets to vessel wall at site of injury. Coagulation/thrombogenesis is initiated by release of TF into the circulation either from the activated/damaged endothelium or from activated platelets followed by excitation of coagulation cascade leading to formation of a stable clot that contains a network of polymerized fibrin with aggregated platelets (Fig. 1.1 and Fig. 1.2)[7, 8].

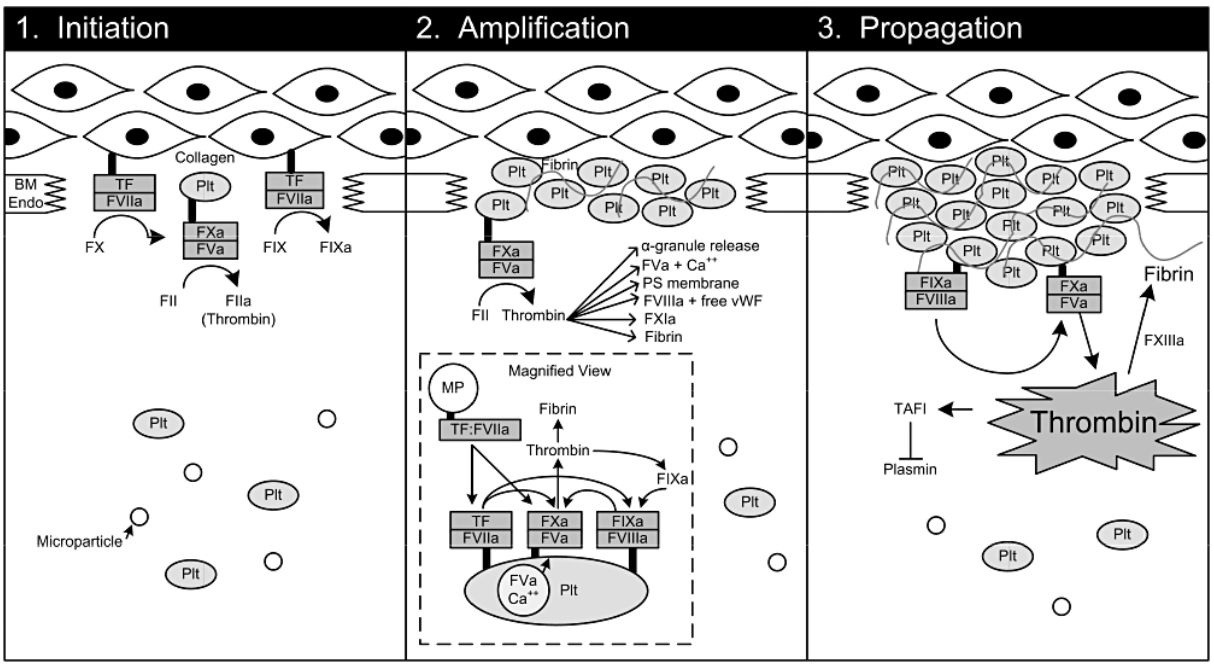

Figure 1.2: The cell-based three phase coagulation model (adapted from Adams and Bird [7]). Figure represents three phases of coagulation. **(1)** Initiation phase: exposure of TF to circulation leads to complexation of TF-FVIIa on plasma membrane of cells within vascular wall (smooth muscle cells and fibroblasts). Small amounts of FIXa, FXa and thrombin are formed due to antagonistic effect of tissue factor pathway inhibitor (TFPI). **(2)** Am plification phase: generation of FIXa:FVIIIa and FXa:FVa complexes on the plasma membrane surface of Plt results into a thrombin burst. **(3)** Propagation phase: the thrombin burst leads to generation and stabilization of fibrin blood clot.
BM: basal membrane; Endo: endothelium; Plt: blood platelet; PS: phosphatidyl serine, TF: tissue factor; *vWF*: von Willebrand factor.

During *initiation phase* generation of thrombin is limited by formation of inactive TF:FVIIa:FXa:TFPI complex resulting in very low concentration of thrombin. However, generation of this little amount of thrombin is sufficient enough to activate FV and FVIII to form FIXa:FVIIIa and FVa:FXa complex essential for thrombin burst and to push the coagulation process into *amplification phase*. Co-localized FXa:FVa and FIXa:FVIIIa complexes on plasma membrane of blood platelets in the presence of Ca^{++} as well as FIX-mediated positive feed-back of thrombin on self generation significantly amplifies the thrombin generation. Generated thrombin is now able to further activate FIX and to release more FVIIIa from its complex with von Willebrand factor (*vWF*), resulting into stable clot formation. *vWF* is also involved in platelet adhesion and FVIII stabilization. Moreover, thrombin-mediated platelet activation also leads to sensitization of platelet receptor glycoprotein IIb/IIIa (a receptor for fibrinogen and *vWF*) and degranulation of α-granules which are responsible for enhanced

platelet aggregation and thus acceleration of the clot formation. Finally, during the *propagation phase* more platelets are conscripted to the site of injury to provide the proper localization of vital components like Ca^{++}, intrinsic tenase complex, prothrombinase complex and a phospholipid surface for efficient thrombin generation. Thrombin converts fibrinogen into fibrin and also activates FXIII which can be covalently crosslinked to fibrin strands. This results into generation of stable insoluble fibrin blood clot. In addition, activation of Thrombin Activatable Fibrinolysis Inhibitor (TAFI) also protects the clot from plasmin-mediated degradation [7, 9].

1.1.2. The fibrinolytic system

Fibrinolysis is initiated, when plasminogen is converted into plasmin by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), present in the blood. In response to venous occlusion, endothelial cells release t-PA which is primarily involved in the dissolution of fibrin blood clot. u-PA is synthesized in a variety of cell-types including monocytes/macrophages, trophoblasts and epithelial cells and is mainly involved in pericellular proteolysis. Proteolytic cleavage of fibrin by plasmin generates C-terminal lysine residues which work as a cofactor for the plasminogen-plasminogen activator complex, thereby stimulating plasmin generation and augmenting fibrinolysis (Fig. 1.1). This positive feedback mechanism is regulated by activated thrombin activatable fibrinolysis inhibitor (TAFIa) which removes C-terminal lysine residues from the surface of partially degraded fibrin, thereby abolishing its cofactor function, impeding plasmin formation and attenuating fibrinolysis. Plasminogen activation is also regulated by Plasminogen Activator Inhibitors-1 (PAI-1) and α_2-antiplasmin (α_2-AP). PAI-1 directly inhibits t-PA and u-PA. α_2-AP forms an inactive complex with free circulating plasmin, whereas fibrin-bound plasmin remains protected from inhibition by α_2-AP [7, 10-12]. Although human and mouse share a conserved fibrinolytic system, however, some interspecies differences exists. Higher resistance of mouse clots to lysis with t-PA compared to human clots is probably due to a shorter plasma half-life of mouse t-PA (6.5 min vs. 80 min for mouse and human t-PA, respectively). Resistance of mouse plasminogen to t-PA-mediated activation is also observed [13].

1.1.3. Thrombosis

Cardiovascular diseases (CVDs) are the leading cause of death worldwide (17.3 million deaths annually; 30% of total deaths worldwide) [14]. Myocardial infarction (MI), stroke and venous thromboembolism (VTE) are ranked among the top three leading cause of death associated with CVDs and are the consequences of thrombosis. Thrombosis is the formation of localized blood clot i.e., thrombus due to excessive coagulation/thrombogenesis in the artery (arterial thrombosis: MI and strokes) or in the vein (venous thrombosis: deep vein thrombosis and pulmonary embolism) [1]. The formation of a localized thrombus results into a blockage of artery or vein and thus prevents blood flow to the organ, leading into cell necrosis and loss of organ function. Therefore, thrombosis represents a pathological condition. Arterial thrombi/white thrombi are formed near unstable atherosclerotic plaque (composed of foam cells and lipids) after rupturing of endothelium and are mainly composed of

platelets. Venous thrombi/red thrombi are a result of alterations in blood vessel, blood flow and/or blood composition and mainly composed of fibrin and red blood cells [1, 3, 4, 7, 8].

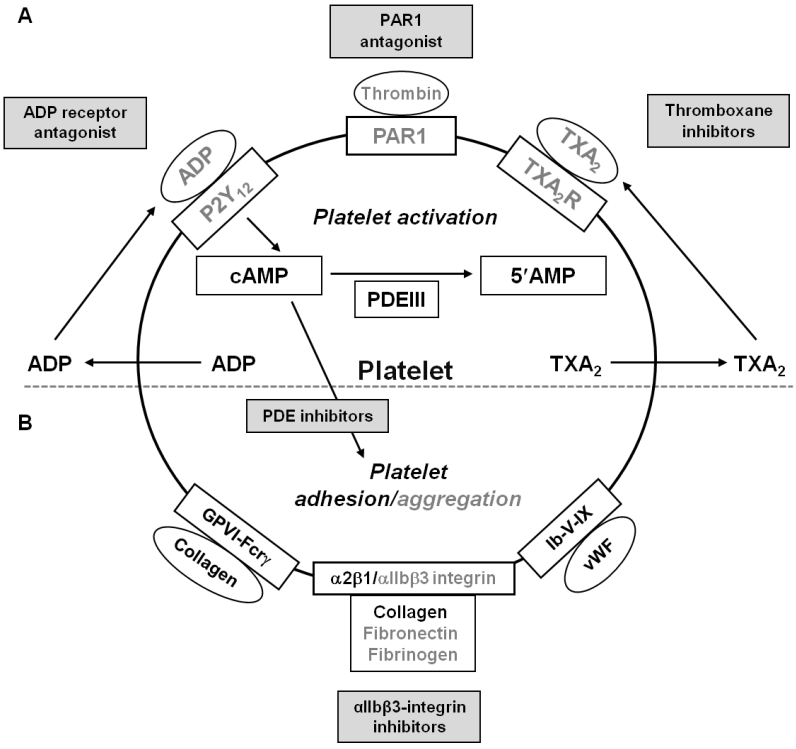

Figure 1.3: Targets of antiplatelet drugs (adapted from Mishra *et al.* [6]). Platelet activation (panel A) and adhesion/aggregation (panel B) is mediated by a variety of cell-surface receptors (black rectangles on cell membrane). The ligands for these receptors are shown in front of them (oval or square). Targets of antiplatelet drugs include ADP receptor $P2Y_{12}$, protease-activated receptor 1 (PAR1), thromboxane A_2 (TXA_2), cyclic nucleotide phosphodiesterase III (PDEIII) and αIIbβ3-integrin.

The currently available antithrombotic therapy targets coagulation by inhibition of platelet activation/aggregation, by inhibition of coagulation factors and/or by stimulation of fibrinolysis by profibrinolytics. Antiplatelet drugs target platelet activation (thromboxane inhibitors; Aspirin and ADP receptor antagonist; Clopidogrel) and platelet aggregation (αIIbβ3-integrin inhibitors or GpIIb/IIIa inhibitors; abciximab) (Fig. 1.3). Anticoagulant therapy is mainly used for the management of venous thrombosis. Vitamin K antagonists (warfarin), heparins, direct FXa inhibitors (rivaroxaban) or direct thrombin inhibitors (Argatroban and Dabigatran) are the main drugs used in this class (Fig. 1.1). Plasminogen activators like streptokinase and genetically modified t-PA (alteplase, reteplase, tenecteplase) are used as thrombolytic or profibrinolytic drugs for treatment of acute thrombotic events. However, timing of intercession i.e. within 4.5 hours after the onset of symptoms for MI and stroke is a critical factor for the success of the therapy. Infusion of t-PA is often associated with bleeding complications. [1, 7, 8, 15, 16].

1.2. Thrombin Activatable Fibrinolysis Inhibitor (TAFI)

Thrombin activatable fibrinolysis inhibitor (TAFI) has been defined as an important regulator of fibrinolysis and is polyonymous in literature: plasma procarboxypeptidase B (proCPB), arginine procarboxypeptidase R (proCPR) and unstable procarboxypeptidase U (proCPU) as identified by independent research groups about 20 years ago [17-21].

1.2.1. Properties of TAFI

1.2.1.1. TAFI synthesis and distribution

In humans, TAFI is encoded by *CPB2* gene which is located on chromosome 13 (13q14.11) and spans ~48 kb of genomic DNA. The *CPB2* gene contains 11 exons which transcribes into a 1.7 kb long transcripts encoding 423 amino acids [22, 23]. However, alternative splicing of mRNA encoding TAFI can also synthesize intracellular proteins without TAFIa activity in different cell types [24]. Nineteen single nucleotide polymorphisms (SNPs) have been recognized in the *CPB2* gene, out of which only six SNPs are located in the coding region of TAFI. Out of these six SNPs only two (+505G/A and +1040C/T) of them resulted in an amino acid substitution corresponding to a 147Ala→Thr and 325Thr→Ile polymorphism, respectively, resulting in four TAFI isoforms (TAFI-A^{147}-T^{325}, TAFI-A^{147}-I^{325}, TAFI-T^{147}-T^{325} and TAFI-T^{147}-I^{325}) [25].

Human TAFI is a prepropeptide of 423 amino acids, which is separately synthesized in the liver and megakaryocytes as indicated by the differences in their glycosylation pattern [19, 26-28]. After the cleavage of the 22 amino acid signal peptide (Met^{1}-Ala^{22}), a 401 amino acid TAFI propeptide (Phe^{1}-Val^{401}) is secreted into the circulation as the highly glycosylated 56 kDa plasma zymogen. In blood, two different pools of TAFI, i.e. the plasma pool (liver-derived) and the platelet pool (megakaryocytes-derived) are present. The platelet pool is only secreted from α-granules upon platelet activation and contributes only 0.1% (50ng/10^9 platelets) of the total amount of TAFI present in the plasma. However, the concentration of TAFI inside the platelet is of the same order of magnitude as that in the plasma. Platelet-derived TAFI and liver-derived TAFI have comparable biochemical and enzymatic properties related to its activation, TAFIa instability and inhibition by TAFIa inhibitors except the glycosylation pattern which is also reflected in the difference between their molecular mass (50 kDa *vs.* 56 kDa for platelet- and plasma-derived TAFI, respectively). Liver-derived TAFI contains five potential N-glycosylation sites (Asn^{22}, Asn^{51}, Asn^{63}, Asn^{86} and Asn^{219}) which except for Asn^{219} are always glycosylated [29]. In plasma, TAFI circulates at a concentration range between 73-275 nM which corresponds to 4-15 µg/ml [30, 31]. The large variation in plasma concentration of TAFI might be the result of (a) the effect of some SNPs on mRNA stability and TAFI gene expression, (b) non-genetic factors (cytokines, hormones and diseases) involved in TAFI gene expression and (c) the different reactivity of the 325Thr→Ile polymorphism in different commercially available ELISAs [25, 32, 33].

A recent study also reported the presence of *CPB2* mRNA in various human non-hepatic cell types (macrophages, megakaryocytes, monocytes, human peripheral blood mononuclear cells and arterial and venous endothelial cells) along with hepatic cells. However, no TAFI expression was detected in these cells except for macrophages [27]. Alternative splicing of mRNA encoding TAFI may be an explanation for the presence of *CPB2* mRNA without TAFI expression in various cell-types [24].

Human and mouse TAFI not only share 85% amino acid sequence identity but also show conservation of important residues involved in substrate binding, glycosylation and zinc binding. Both TAFI have similar biochemical characteristics with respect to their activatability and their antifibrinolytic effect. TAFI levels in mouse plasma are 2-6 µg/ml,which is comparable to those in human plasma. Therefore, it can be concluded that TAFI pathways are conserved in human and mouse and conclusions depicted from mouse models can in most cases be extrapolated to humans [34-36].

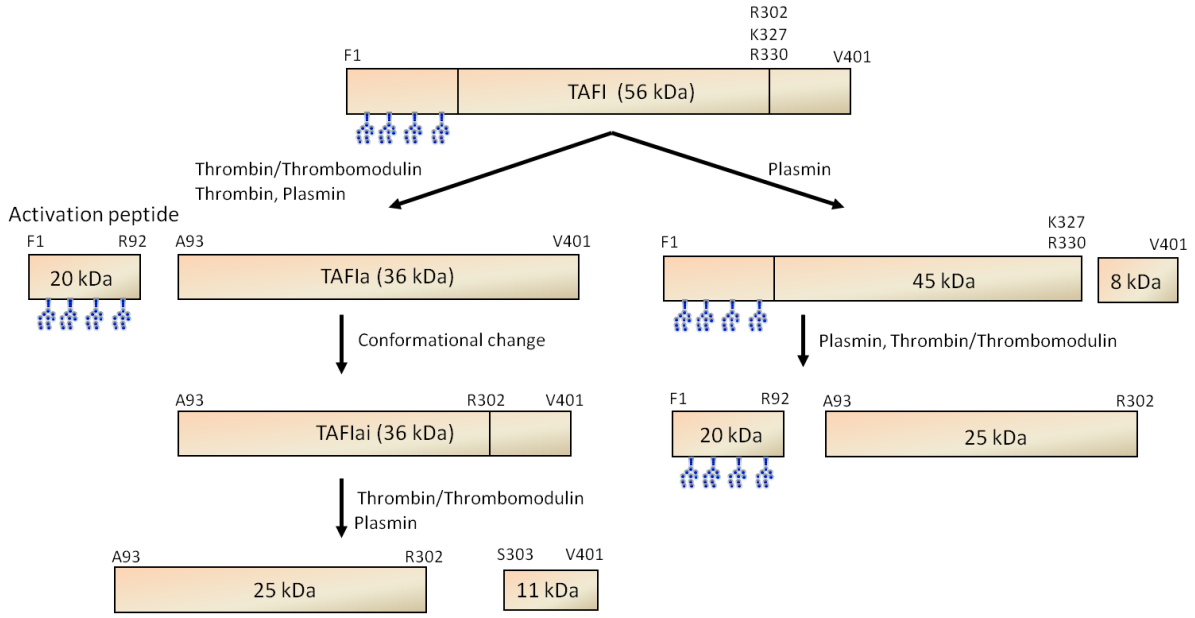

Figure 1.4: TAFI activation and inactivation (adapted from Marx *et al.* [37]). TAFI can be activated by thrombin, the T/TM complex or plasmin by proteolytic cleavage at Arg^{92}, resulting in the release of highly glycosylated activation peptide (20kDa) and generation of TAFIa (36kDa). TAFIa reveals an intrinsic temperature dependent instability and converted into inactive TAFIai due to conformational changes. TAFIai is further proteolytically cleaved into two fragments of 25kDa and 11kDa each. Alternatively, plasmin can also cleave TAFI at Lys^{327} and Arg^{330}, resulting in a truncated inactive form of TAFI (45 kDa) which can be further cleaved at Arg^{92} and Arg^{302} leading to the generation of two fragments of 25kDa and 20kDa each.

1.2.1.2. TAFI activation and TAFIa instability

TAFI can be proteolytically activated by trypsin-like serine proteases such as thrombin, the thrombin-thrombomodulin (T/TM) complex and plasmin *in vitro* by cleavage at Arg^{92} [38, 39] (Fig. 1.4), resulting into the release of the activation peptide (Phe^{1}-Arg^{92}, 20kDa) from TAFIa (Ala^{93}-Val^{401}, 36 kDa) which allows access of large macromolecular substrate like fibrin to the active site of the enzyme [40]. While thrombin promptly activates TAFI, the T/TM complex activates TAFI with a 1250-fold higher catalytic efficiency compared to thrombin alone [38, 41] and therefore the T/TM complex is supposed to be the most important physiological activator of TAFI as suggested by some *in vitro* [42, 43] and one *in vivo*

analysis [44]. However, the T/TM complex could only activate TAFI at the endothelium as TM is only located on the surface of endothelial cells [45, 46]. However, often the fibrin-rich clot, where TAFIa regulates fibrinolysis extends away from the endothelium. Since, only very small amounts of TAFIa are needed to attenuate fibrinolysis [20, 47], the contribution of other TAFI activators in the regulation of fibrinolysis within the clot cannot be precluded. Plasmin was also shown to activate TAFI *in vitro* and is a stronger activator of TAFI than thrombin [37, 39]. In presence of glycosaminoglycan like heparin the catalytic efficiency of plasmin increased 16-fold which still remained 10-fold lower than the T/TM complex.

TAFIa is an unstable enzyme with half life ($t_{1/2}$) of 8 and 15 min at 37°C depending on the polymorphism at position 325 (Thr^{325} *vs.* Ile^{325}) [48, 49]. The half-life of TAFIa also correlates with its anti-fibrinolytic effect [49, 50]. The antifibrinolytic effect of TAFIa is regulated by its intrinsic thermal instability [17, 51] which results in inactivation TAFIa into TAFIai due to conformational changes. The induced conformational changes also make the catalytic domain more vulnerable to proteolysis by thrombin, the T/TM complex and plasmin at Arg^{302} which is further cleaved into two fragments of 25 kDa and 11 kDa (Fig. 1.4). In addition, plasmin can also cleave TAFI at Lys^{327} and Arg^{330} and therefore a truncated ~45 kDa inactive form of TAFI can also be generated. The activation peptide from this truncated form can still be cleaved by thrombin, the T/TM complex or plasmin, but this fragment will no longer be able to form an active fragment. Several mutations that enhance the half-life of the TAFIa have been identified [48, 49, 52, 53] and a TAFI-ACIIYQ variant (TAFI-A147-S305C-T325I-T329I-H333Y-H335Q) was engineered with ~180-fold increased TAFIa half-life [53] (Fig. 1.5B). The enhanced thermal stability of the enzyme also reflects into the increased functionality of the enzyme.

1.2.1.3. TAFI structure

TAFIa belongs to the zinc metallocarboxypeptidase A family of proteins (TAFIa; EC 3.4.17.20) [19, 54]. Using the search command thrombin activatable fibrinolysis inhibitor in the protein data bank (PDB) 24 structural hits were retrived. Out of which only five entries were for human TAFI/ human tissue procarboxypeptidase B whereas the others were related to bovine TAFI and porcine pancreatic carboxypeptidase B. Out of five PDB entries 1KWM was for human tissue procarboxypeptidase B which shares 42% sequence identity with human TAFI and TAFI structure was simulated from human tissue procarboxypeptidase B. Rest four entries were for human TAFI (Table 1.1). The latest structure (3LMS) from by Sanglas L *et al*. (2010) was for TAFIa and not for intact TAFI and entry 3D67 was for intact TAFI in complex with GEMSA. Superimposition of 3D67 and 3LMS on 3D66 (intact TAFI) do not reveal any deviation from the structure 3D66. Since throughout the thesis intact TAFI is refered, therefore, 3D66 is used as a representative structure of TAFI in the thesis.

The intact TAFI can structurally be divided into two domains : (1) highly glycosylated N-terminal activation peptide (Phe^{1}-Arg^{92}, 20kDa) composed of two α-helices and four β-strands (Phe^{1}-Val^{76}) with a partially helical linker (Glu^{77}-Arg^{92}) and (2) the catalytic domain (Ala^{93}-Val^{401}; 36 kDa), composed of a central β-sheet surrounded by nine α-helices [55] (Fig. 1.5A). Glycosylation (9.4 kDa) contributes approximately to 50% and 17% of the molecular weight of the activation peptide and the

intact TAFI, respectively. Three disulphide bonds (Cys^{156}-Cys^{169}, Cys^{228}-Cys^{252} and Cys^{243}-Cys^{257}) presented in the catalytic region are responsible for intra-chain linkage [56]. The catalytic domain also comprises the dynamic flap (Phe^{296}-Trp^{350}) which is responsible for instability of TAFI. The crystal structure of TAFI reveals the existence of hydrophobic interactions between the dynamic flap (Tyr^{341}) and the activation peptide (Val^{35} and Leu^{39}) which stabilize TAFI in its intact conformation (Fig. 1.5A) [55]. TAFI activation leads to disruption of these stabilizing interactions and hence results in the enhancement of the mobility of the dynamic flap region, which induces an irreversible conformational change in TAFIa. This not only leads to the disruption of the catalytic site (TAFIai) but also to the exposure of the thrombin and the plasmin cleavage site (Arg^{302}) and consequently to further proteolytic degradation of TAFIai (Fig. 1.4). In the catalytic pocket, the catalytic Zn^{++} is coordinated by residues His^{159}, Glu^{162} and His^{288} (Fig. 1.5B). Residues Asn^{234}, Arg^{235} and Tyr^{341} are important for substrate binding and residues Arg^{217} and Glu^{363} are involved in hydrolysis.

Table 1.1: Summary of all the TAFI structures reported in PDB.

Structure	Ligand	PDB ID	Resolution	Author	Year
TAFIa	TCI	3LMS	2.5 Å	Sanglas *et al.* [203]	2010
TAFI wt	–	3D66	3.1 Å	Brondijk *et al.* [55]	2008
TAFI	GEMSA	3D67	3.4 Å	Brondijk *et al.* [55]	2008
TAFI-IIYQ	–	3D68	2.8 Å	Brondijk *et al.* [55]	2008

Tick-derived funnelin inhibitor, TCI

1.2.2. (Patho-)physiological functions of TAFI

TAFIa has distinct biochemical characteristics and therefore is involved in a wide range of physiological processes. Recent studies revealed that along with fibrin, TAFIa also has a substrate specificity towards anaphylatoxins (C3a, C5a), annexin II, bradykinin, and thrombin cleaved osteopontin and thus indicating its possible role in blood pressure, cell migration, inflammation and sepsis [25, 57-61]. However, the best studied physiological function of TAFI is its regulatory role in fibrinolysis.

1.2.2.1. TAFI as an antifibrinolytic agent

Fibrinolysis starts when t-PA and/or u-PA converts inactive plasminogen into active plasmin, a major component of fibrinolysis. Upon partial degradation of fibrin by plasmin, the C-terminal lysine residues of fibrin get exposed on the surface of the fibrin blood clot and stimulate further plasmin generation by: (a) increasing the affinity of plasminogen for partially degraded fibrin leading to enhancement of plasmin generation, (b) stimulating plasmin to convert Glu-plasminogen to Lys-plasminogen which is a superior substrate for t-PA, and (c) protecting plasmin from inactivation by α_2-AP (Fig. 1.6). However, TAFIa removes these lysine residues from fibrin blood clot and regulates fibrinolysis through a negative feedback mechanism [62].

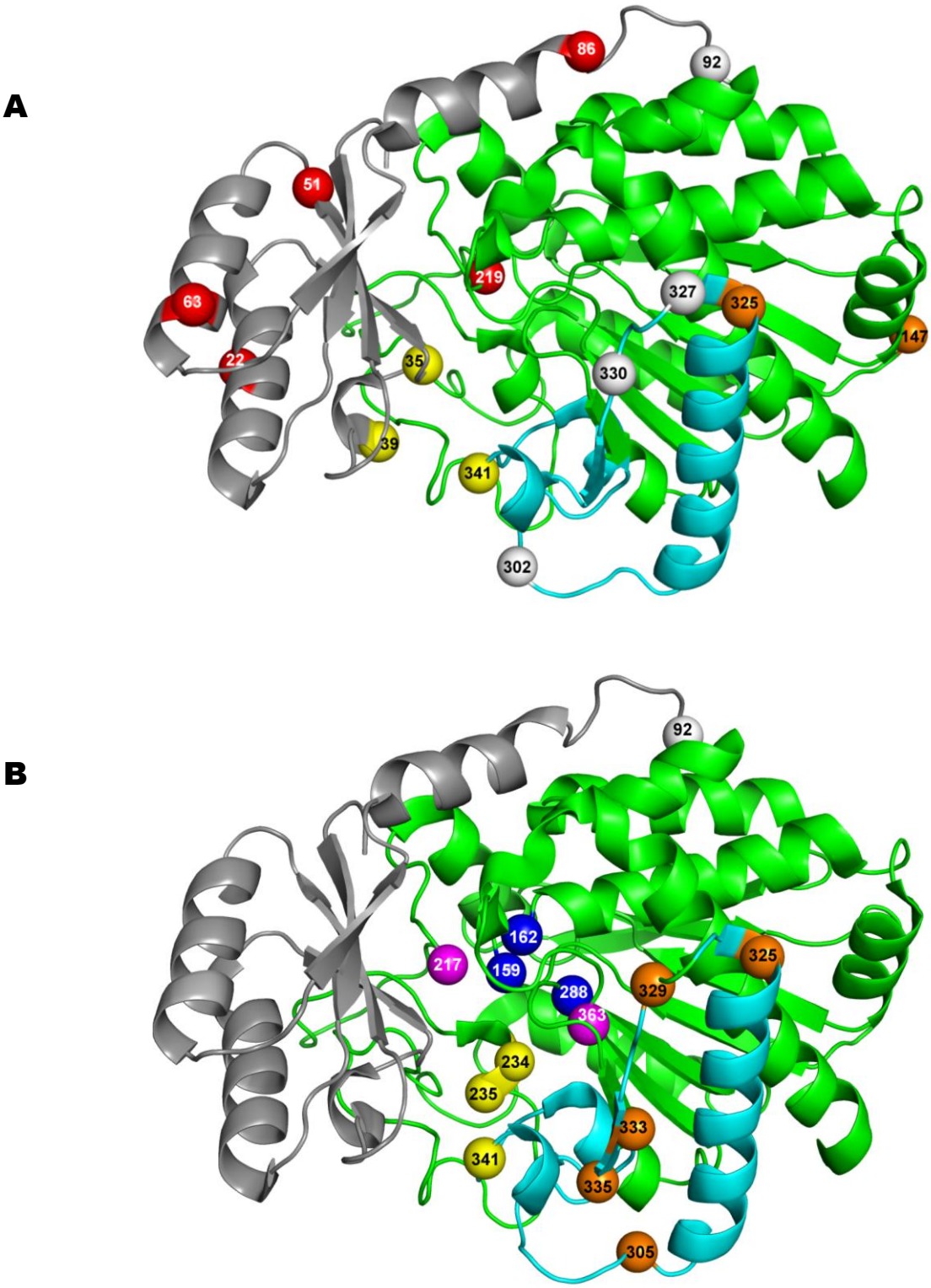

Figure 1.5: Ribbon diagram of the human TAFI structure (based on the crystal structure of Marx *et al.* [55]). The activation peptide, the catalytic domain and the dynamic flap region (residues 296-350) is shown in grey, green and cyan, respectively. (A) The thrombin (Arg^{92} and Arg^{302}) and the plasmin (Arg^{92}, Arg^{302}, Lys^{327} and Arg^{330}) cleavage sites are shown as white spheres. Residues involved in the glycosylation (Asn^{22}, Asn^{51}, Asn^{63}, Asn^{86} and Asn^{219}), polymorphisms (Ala/Thr^{147} and Thr/Ile^{325}) and interactions between the dynamic flap (Tyr^{341}) and the activation peptide (Val^{35} and Leu^{39}) are depicted as red, orange and yellow spheres, respectively. (B) Residues important for (in)stability (Ser^{305}, Thr^{325}, Thr^{329}, His^{333}, His^{335}), hydrolysis (Arg^{217} and Glu^{363}), substrate binding (Asn^{234}, Arg^{235} and Tyr^{341}) and zinc binding (His^{159}, Glu^{162} and His^{288}) are shown in orange, magenta, yellow and blue spheres, respectively.

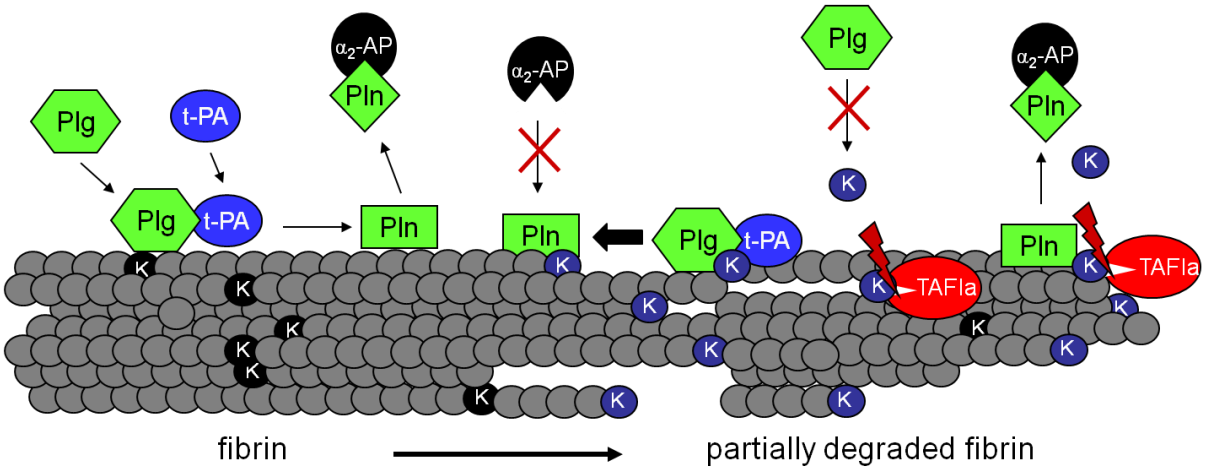

Figure 1.6: TAFIa in regulation of fibrinolysis (adapted from Leurs and Hendriks [63]). Binding of plasminogen (Plg) and t-PA to the internal lysine residues (K) on the surface of fibrin blood clot leads to the generation of plasmin (Pln). α_2-AP inhibits the free circulating plasmin but fail to inhibit fibrin bound plasmin. The fibrin bound plasmin starts degradation of the fibrin clot resulting in the exposure of more C-terminal lysine residues (K) which act as a cofactor for plasminogen-t-PA complex resulting into a plasmin burst. TAFIa abolishes this cofactor function by removal of the C-terminal lysine residues ( ) and thereby downregulates fibrinolysis.

Regulation of fibrinolysis by TAFIa is a threshold-dependent phenomenon as fibrinolysis is only abrogated till the concentration of TAFIa is above a particular threshold level [64]. As soon as the concentration of TAFIa goes below this level, exponential increase in C-terminal lysine residues on fibrin takes place which boosts plasmin formation and speeds up fibrinolysis. The critical threshold level of TAFIa is directly proportional to the plasmin concentration which is dependent on the rate of plasminogen activation and therefore correlated with the concentrations of t-PA and plasmin inhibitors (α_2-antiplasmin, α_2-macroglobulin and antithrombin). The duration above the threshold concentration of TAFIa is also associated with the plasma TAFI concentration, the extent of its activation, and its stability. A very small amount of TAFIa (1-5 nM, < 2% of the total plasma concentration of TAFI) is enough to attenuate fibrinolysis and therefore a slow and continuous rate of TAFI activation should provide a better regulation of fibrinolysis than a massive but short-lived burst of TAFIa [64, 65].

The TAFI zymogen also exhibits carboxypeptidase activity which has a 41-fold lower catalytic rate and a 2-fold higher substrate affinity, resulting into an 18-fold lower catalytic efficiency than TAFIa [66]. However, the contribution of TAFI zymogen in the down-regulation of fibrinolysis is a matter of debate. The prolongation of clot lysis by the TAFI zymogen in a t-PA-induced clot lysis assay in presence of TAFIa inhibitors (potato tuber carboxypeptidase inhibitor; PTCI or guanidinoethyl-mercaptosuccinic acid; GEMSA) was previously demonstrated [67], suggesting that the TAFI zymogen can regulate fibrinolysis. However, in another study using snake venom batroxobin as an alternative for thrombin for clot induction, but without TAFI activation properties, no prolongation in t-PA-induced clot lysis was observed indicating no role of the TAFI zymogen in the regulation of fibrinolysis [68]. The contradicting results (PTCI or GEMSA *vs.* batroxobin) may be attributed to the differences in the clot induction in both experiments [67]. Later it was proposed that due to limited accessibility of the

catalytic cleft of the TAFI zymogen, TAFI can only cleave efficiently small substrates but no longer substrates like plasmin-modified partially degraded fibrin [40].

1.2.2.2. Role of TAFI in thrombotic diseases

Some epidemiological studies revealed a positive correlation between elevated plasma TAFI levels and increase in the risk of thrombotic tendencies [69, 70] while some contradicted these findings i.e. reported no or even a negative correlation [63, 71-74]. This can be explained by the fact that the majority of these studies used poorly characterized activity based or antigen-based (ELISAs) assays without including generalized standards/calibrator and reference samples in their assays. Due to different reactivity of the different TAFI isoforms, ELISAs used in these studies were also not able to measure TAFI concentrations accurately [33]. Furthermore, application of well characterized assays which can differentiate among the activation peptide, TAFIa and total TAFI seem to correlate TAFIa/ activation peptide concentrations with thrombotic events [75-77] .

In addition to the epidemiological studies, both genetic and pharmacological inhibition approaches have also been used to investigate the *in vivo* role of TAFI in different thrombosis models. Studies revealed that TAFI deficient mice (TAFI KO) have a comparable phenotype as wild-type (WT) mice and showed only an enhanced fibrinolysis in *ex vivo* rotational thromboelastometry and in an *in vivo* mouse thromboembolism model [78]. Several studies using TAFI KO mice like a batroxobin-induced pulmonary embolism model,which showed a reduced accumulation of I^{125}-fibrin in the lungs of TAFI KO mice [79] and a $FeCl_3$-induced thrombosis model which illustrated reduction in thrombus size and weight [80] correlated TAFI deficiency with fibrinolytic enhancement. Similarly, pharmacological inhibition of TAFI using the monoclonal antibody (MA) MA-TCK26D6 in a mouse thromboembolism model also revealed a decreased fibrin deposition in the lungs of mice upon administration of MA-TCK26D6 [81]. However, in a photochemical injury model of arterial thrombosis no difference concerning time to thrombus formation as well as rate of spontaneous reperfusion due to a reduction in thrombus size was observed in TAFI KO and WT mice [82]. Moreover, in different other thrombosis models also intravenous injection of various thrombotic agents such as factor X, LPS and thrombin etc. have not resulted in significant increase in survival rate or significant reduction in fibrin deposition in lungs of TAFI KO mice than WT mice [82]. These contradicting results in different models may be ascribed to the differences in the nature of the agents used to stimulate thrombogenesis, their strength as well as the spatial localization of thrombus. However, despite different contradictory results in different thrombosis models the general conclusion inclined towards an improved fibrinolytic activity upon TAFI inhibition (Table 1.2) [60, 83].

1.2.3. TAFI inhibition

1.2.3.1. Direct inhibition of TAFIa or prevention of TAFI activation

TAFI/TAFIa [TAFI(a)] is a putative target to develop profibrinolytic drugs. No physiological inhibitors of TAFI(a) are reported till date. Immense research have been carried out to discover TAFI(a) inhibitors. TAFIa can be inhibited by chelating agents (o-phenantroline and EDTA), reducing agents

(dithiothreitol and 2-mercaptoethanol) and small synthetic substrate analogues like MERGETPA (DL-2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid), GEMSA (guanidinoethyl-mercaptosuccinic acid) and ε-aminocaproic acid (ε-ACA) [63]. However, a cross-reactivity for other plasma carboxypeptidases like CPN and low oral bioavailability due to high polarity are the two main disadvantages of these small substrate inhibitors [62]. Efforts have been made to identify synthetic TAFIa inhibitors with higher potency, favourable pharmacokinetic properties (absorption, distribution and clearance) and less cross-reactivity toward CPN [62, 84]. However, interaction with pancreatic carboxypeptidase B remained associated with these synthetic inhibitors. Naturally occurring TAFIa inhibitors with high selectivity for TAFIa such as potato tuber carboxypeptidase inhibitor (PTCI), leech carboxypeptidase inhibitor (LCI) and tick carboxypeptidase inhibitor (TCI) have also been identified. However, the biphasic effect of some reversible TAFIa inhibitors (PTCI, GEMSA and a nanobody) in *in vitro* clot lysis eclipsed their benefits [85, 86]. The biphasic effect of reversible TAFIa inhibitors can be defined as their ability to enhance fibrinolysis at higher concentrations but to prolong clot lysis at lower concentrations. The antifibrinolytic effect of these inhibitors at low concentrations can be attributed to their capability to stabilize TAFIa [85, 87]. In the presence of low concentrations of a reversible inhibitor, when free TAFIa is irreversibly inactivated to TAFIai, the bound form that is protected against inactivation replenish the plasma pool due to shift in the equilibrium whereas at higher concentrations of inhibitor the equilibrium is driven toward its bound form resulting in the reduction of free TAFIa levels below the threshold.

Alternatively, MA have been established as candidate drug molecules in different disorders with high potency, selectivity and desired pharmacokinetic properties. To prevail over the problem of non-selectivity of several TAFI inhibitors, various extremely selective monoclonal antibodies (MA) and nanobodies either inhibiting TAFI activation by different mechanisms or directly interfering with TAFIa activity have been produced [44, 86, 88-90]. MA-T9H11 and MA-RT30D8 directly inhibit human or rat TAFIa [88, 89] whereas MA-TCK26D6 inhibits plasmin-mediated activation of human, rat and mouse TAFI [81]. MA-T12D11 and mAbTAFI/TM#16 selectively inhibit T/TM-mediated human TAFI activation [44, 88]. Furthermore, to reduce immunogenicity of mouse antibodies in humans single-chain variable fragments (scFvs) derived from MA against TAFI were constructed, which in most cases revealed similar inhibitory properties as their parental MA [91].

1.2.3.2. *In vivo* TAFI regulation

Recent *in vivo* studies suggest that the use of TAFI(a) inhibitors as an adjuvant therapy next to the standard thrombolytic drugs can potentially reduce the therapeutic dose of thrombolytic drugs and thereby the risk of associated bleeding. Some *in vivo* studies reported no positive effect of genotypic (TAFI KO) or pharmacological (PTCI) inhibition of TAFI in various arterial and venous thrombosis models (Table 1.2) [78, 80, 82, 92, 93]. However, several *in vivo* studies reported an enhanced fibrinolysis in TAFI KO mice (Table 1.2). Similar results were also observed in various arterial and venous thrombosis models during pharmacological inhibition of TAFI in combination with low t-PA concentrations (exogenous fibrinolysis) [78, 81, 92, 94-97]. However, for the prevention of thrombotic events the application of TAFI inhibitors as single therapeutics to enhance endogenous fibrinolysis is

under debate. In various arterial and venous thrombosis models administration of TAFI(a) inhibitors reported to enhance endogenous/spontaneous fibrinolysis [44, 79, 80, 93, 94, 98-102]. MA-TCK26D6 which inhibits plasmin-mediated activation of TAFI and mAbTAFI/TM#16 which inhibits T/TM-mediated activation of TAFI have been shown to accelerate fibrinolysis when administered in mouse thromboembolism model and *E.coli*-induced sepsis model in baboons, respectively [44, 81]. Moreover, increased plasmin-(α_2-AP) levels in plasma of thromboembolism-induced mice in presence of MA-TCK26D6 also suggest an augmented fibrinolysis [81]. Administration of PTCI even after thrombus formation was also able to increase fibrinolysis in a $FeCl_3$–induced vena cava mouse thrombosis model but only in mild thrombogenic conditions [93]. Therefore, it can be concluded that TAFIa regulates fibrinolysis in *in vivo* arterial and venous thrombosis models and that the efficiency of TAFI inhibitors relies on the thrombogenic stimulus, localization of thrombogenesis and timing of intervention.

Various companies have patented a variety of small molecule TAFIa inhibitors for the management of various pathological conditions associated to TAFI inhibition and some of them are already in different phases of clinical trials [103]. However, the potency, specificity, toxicity and bioavailability of a TAFI(a) inhibitor in clinical settings are still a concern. Based on limited information available related to the clinical application of TAFI(a) inhibitors, it can be said that more potential candidates are required to further proceed in clinical trials.

Table 1.2: Different thrombosis models used to evaluate the role of TAFI deficiency/inhibition in fibrinolysis.

Animal	Model	Fibrinolysis	Inhibitors	Parameters	Effect	Ref
A: No effect of TAFI deficiency or pharmacological inhibition on fibrinolysis						
TAFI KO vs. WT mouse	3.5% $FeCl_3$-induced carotid artery thrombosis	Endogenous	–/PTCI	Blood flow	–	[80, 93]
	Rose Bengal induced carotid artery thrombosis	Endogenous	–	Occlusion time	–	[82]
	Rose Bengal induced jugular vein thrombosis	Endogenous	–	Occlusion time	–	[82]
	Tail bleeding	Endogenous	–	Blood loss	–	[82]
	Thrombin-induced thromboembolism	Endogenous	–	Survival rate	–	[82]
	Factor X-induced thrombosis	Endogenous	–	Survival rate and kidney fibrin deposition	–	[82]
	LPS-induced septic shock model	Endogenous	–	Survival rate	–	[82]
	Tail bleeding	Endogenous	–	Bleeding time	–	[78]
	$FeCl_3$-induced mesenteric thrombosis	Endogenous	–	Occlusion time	–	[78]
Rat	Tail bleeding time	Exogenous	PTCI	Bleeding time	–	[92]
B: Positive effect of TAFI deficiency or pharmacological inhibition on fibrinolysis						
Mouse	TF-induced thromboembolism	Exogenous	MA-TCK26D6	Lung fibrin deposition	↓	[81]
TAFI KO vs. WT mouse	Batroxobin-induced pulmonaryembolism	Endogenous	–	I^{125}-Fibrin deposition in lungs	↓	[79]
	Tail bleeding	Endogenous	–/PTCI	Blooding time	↑	[80, 93]
	3.5% $FeCl_3$-induced Vena Cava	Endogenous	–/PTCI	Thrombus weight	↓	[80, 93]
	TF-induced thromboembolism	Exogenous	–	Lung fibrin deposition	↓	[78]
Rat	Laser-induced femoral artery thrombosis	Exogenous	BX528	Reperfusion rate	↑	[94]
	$FeCl_3$-induced arterial thrombosis	Exogenous	PTCI	Occlusion time	↓	[92]
	Batroxobin/LPS-induced lung fibrin deposition	Endogenous	BX528	I^{125}-fibrin deposition in lungs	↓	[94]
	LPS-induced endotoxemia	Endogenous	EF6265	I^{125}-Fibrin deposition in kidney and liver	↓	[98]
	TF-induced microthrombosis	Endogenous	DD2, MERGEPTA and PTCI	Glomerular fibrin deposition	↓	[99, 100]
	Laser-induced mesenteric arterioles	Endogenous	PTCI	Thrombus size	↓	[101]
Rabbit	*Ex vivo* jugular vein model	Exogenous	BX528	Thormbus weight	↓	[94]
	TF-induced Jugular vein thrombolysis	Exogenous	PTCI, 3-Mercaptopropionic acid	Thormbus weight	↓	[95, 96]
	Arterial thrombolysis	Exogenous	PTCI	Reperfusion time	↑	[97]
	Ex vivo jugular vein thrombosis	Endogenous	PTCI	Fibrinolysis	↑	[102]
Dog	$FeCl_2$- induced femoral artery thrombosis	Exogenous	BX528	Reperfusion rate	↑	[94]
Baboon	*E. coli* induced sepsis model	Endogenous	mAbTAFI/TM#16	FDP conc. and loss of fibrinogen	↑	[44]

LPS; lipopolysaccharide and TF; tissue factor.

1.3. Antibodies and Nanobodies

Protein-based biologicals are animal- or microorganism-derived naturally occurring proteins or peptides such as growth hormone and insulin and are among some of the most powerful tools in therapy and diagnostics against infections and diseases [104, 105].

1.3.1. Antibodies/ Immunoglobulins

Antibodies (Abs)/Immunoglobulins (Igs) are the largest, most versatile and fastest growing class of biologicals. In mammals, Abs are produced by B-cells and circulate in the human body through blood. Igs are an integral part of immune system which utilizes Igs to recognize, to bind, to neutralize and to remove foreign antigens such as bacteria, toxins or viruses form the body [106-109].

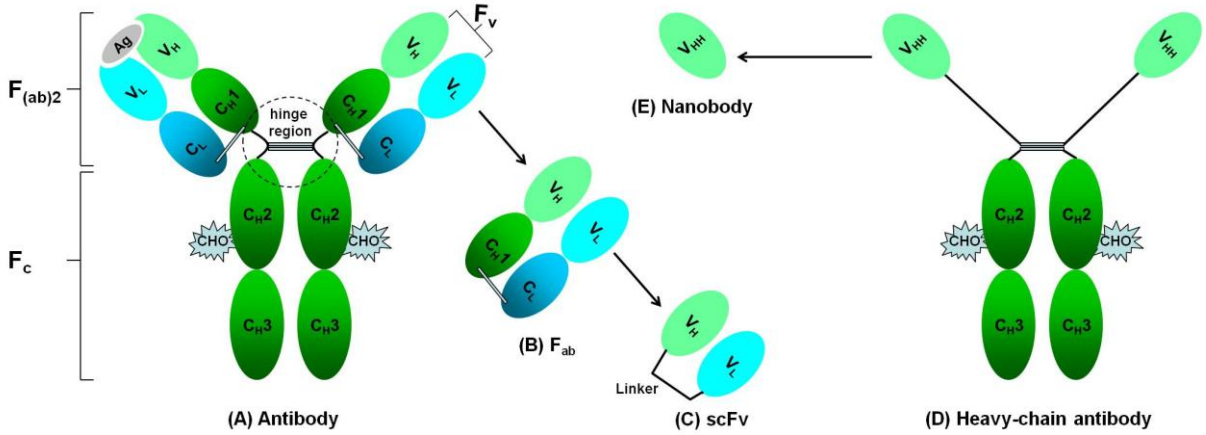

Figure 1.7: Structure of an antibody, and its derivatives (adapted from Holliger and Hudson [110]). (A) Heavy chains and light chains are represented in green and blue. Variable regions (V_H and V_L) are depicted in light green and light blue. The conserved glycosylation is indicated as CHO. Antigen binding is mediated via the Fv part of F_{ab}. **(B)** A Fab fragment is composed of V_L-C_L and V_H-C_H1 and **(C)** a scFv is only one variable region of the heavy (V_H) and light chains (V_L) joined by short linker. **(D)** Heavy-chain antibody (HCAbs) lacks the C_H1 domain as well as the light chains of antibody and **(D)** nanobody represents the variable domain of HCAbs.

1.3.1.1. Structure and function

Abs are Y-shape glycoproteins with a molecular weight of 150 kDa, which are composed of 4 polypeptide chains i.e. two identical heavy chains and light chains, connected via disulfide bonds around a hinge region (Fig. 1.7). Both heavy and light chains contain an N-terminal variable region (V_L or V_H), which together form the fragment variable (Fv) responsible for antigen-binding and several constant domains (C_L or C_H: C_H1, C_H2 and C_H3). C_H1, C_H2 and C_H3 domains of the heavy chain are responsible for catalysis of peroxide formation; complement interaction and Fc-receptor interaction on phagocytes, respectively. The Fv part is composed of three hypervariable regions also known as complementary determining regions (CDRs), because the antigen-binding sites (paratope) are complementary to the epitope on the antigen (Fig. 1.8), which are altered with four framework regions (FR1-4). FRs act as a scaffold (comprising nine β-strands organized in four- and five-stranded β-

sheets) on which CDRs are folded in canonical structures. At the C-terminal of the antibody, the crystallisable fragment (Fc) is located which is responsible for the maintenance of the structure as well as *in vivo* half-life of Igs. Fc also enables binding of Igs to different immunocomponents, such as effector cells or complement factors (C1q) via Fc receptors and mediates its effector functions such as antibody- and complement-dependent cell-mediated cytotoxicity (only IgG and IgM) [106, 108, 111, 112].

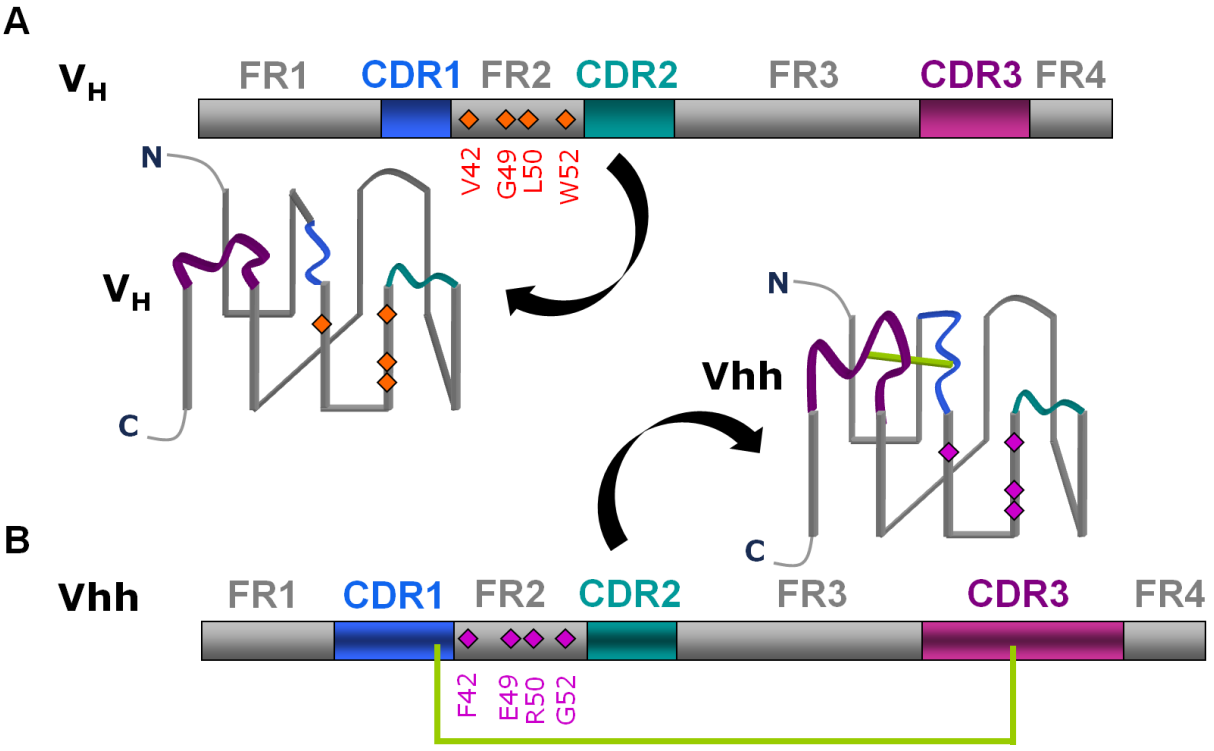

Figure 1.8: Comparison of V_H (A) and Vhh (B) (adapted from Muyldermans [112]). V_H segments of antibody and V_{HH} segment of HCAbs (Vhh)/nanobody, respectively are composed of CDR1 (blue), 2 (green) and 3 (magenta) alternated with framework (FRs: grey). CDRs are folded on FR scaffold to form canonical structure. The hydrophilic substitutions in FR of Vhh are depicted in pink. A Vhh-fragment also has a longer CDR1 and 3 and an additional disulfide bridge between CDR1 and CDR3 (light green).

Human Igs can be classified in five different isoforms (i.e. IgA, IgD, IgE, IgG and IgM), depending on the composition of their heavy chains (i.e. α, δ, ε, γ and μ, respectively) which also determines their size (monomeric *vs*. multimeric; ranging from 150-1150 kDa), antigen binding capability (2-12) and biological functions. Additionally, every Ab consists of either kappa (κ) or lambda (λ) light chains which do not have an impact on the biological function of the Igs. Widely used Abs for therapeutics and diagnostics belong to the most abundant class of Igs i.e. IgG [106-109].

1.3.1.2. Generation of antibodies

In nature, exposure of antigen to the immune system generates two types of protective immune responses i.e. (a) a cell-mediated response and (b) a humoral response. In the humoral response, the antigen works as a stimulus for B cell activation leading to proliferation of a large number of diverse B-cells and generation of diverse antibodies. These different antibodies differ in their affinity, selectivity,

binding site and functionality and in general are termed as polyclonal antibodies. Polyclonal antibodies are naturally produced by inoculating antigens in a suitable mammal like mouse, rabbit or goat and can be purified from the serum of the immunized animal using Prosep-A column. However, high heterogeneity, high non-relevant IgGs, limited supply and batch to batch variations limited its applicability as therapeutic molecule. In contrast, MA are derived from single cell line, therefore are monospecific in nature as it recognizes only one particular epitope and have identical affinity, physiochemical properties and functionality. Furthermore, it can be produced in unlimited amount with high reproducibility. Hybridoma technology is the first developed and most widely used technology to produce MA, described by Galfré and Milstein [113]. It implies the generation of mouse hybridomas by stable fusion of antigen producing B cells obtained after immunization of mice with the target antigen with immortal myeloma cells using polyethylene glycol or electrically induced fusion technique. Once the hybridoma cell line is formed, large amounts of MA can be produced by using celline systems. Hybridoma cell lines can also be cryopreserved for a very long time [108, 113, 114].

1.3.1.3. Therapeutic antibodies

Today MA are the most resourceful and fastest expanding class of drugs with high selectivity, stability and desired pharmacokinetic/phamacodynamic properties. Till date approximately 35 therapeutic MA are in the clinics and used for the treatment of autoimmune diseases, cancer and inflammatory diseases. Hundreds of more MA are in various developmental phases [115, 116]. Initially, antibody therapy started as serum therapy with administration of crude polyclonal in form of serum. Later, the advent and advancement of the hybridoma technology led to the therapeutic application of target-specific MA. However, therapeutic application of these mouse MA was hindered due to their inability to trigger human effector function, short plasma half-life and a human anti-mouse antibody (HAMA) responses. Humanization of mouse MA (chimerization and CDR-grafting) and development of fully human antibodies facilitated to circumvent these problems (Fig. 1.9). In chimerization, the constant domain of mouse MA is exchanged with its human IgG counterparts whilst CDR-grafting involves grafting of the mouse CDRs onto a human Ig scaffold [115, 117, 118]. Currently fully human antibodies can be produced by (a) screening large recombinant antibody libraries constructed by expression of human antibody fragments on bacteriophages, yeasts, mRNAs or ribosomes [119], (b) fusing B cells acquired from transgenic mice carrying human Igs genes with mouse myeloma cells and (c) immortalization of human antibody-secreting cells by Epstein-Barr virus followed by fusion with a human myeloma celline (EBV-hybridoma technique) [120]. Another approach to prevail over the immunogenicity of MA is the production of smaller antibody fragments like Fab and single-chain variable fragments (scFvs) (Fig. 1.7). These fragments revealed in general a similar affinity like the parental MA but with higher tissue penetration. However, low yields and aggregation tendency are the major challenges involved in the production of the antibody fragments [103]. Furthermore, all above mentioned techniques do not result into a complete loss of the immune responses. Therefore, further studies are needed to develop methods to improve antibody therapy.

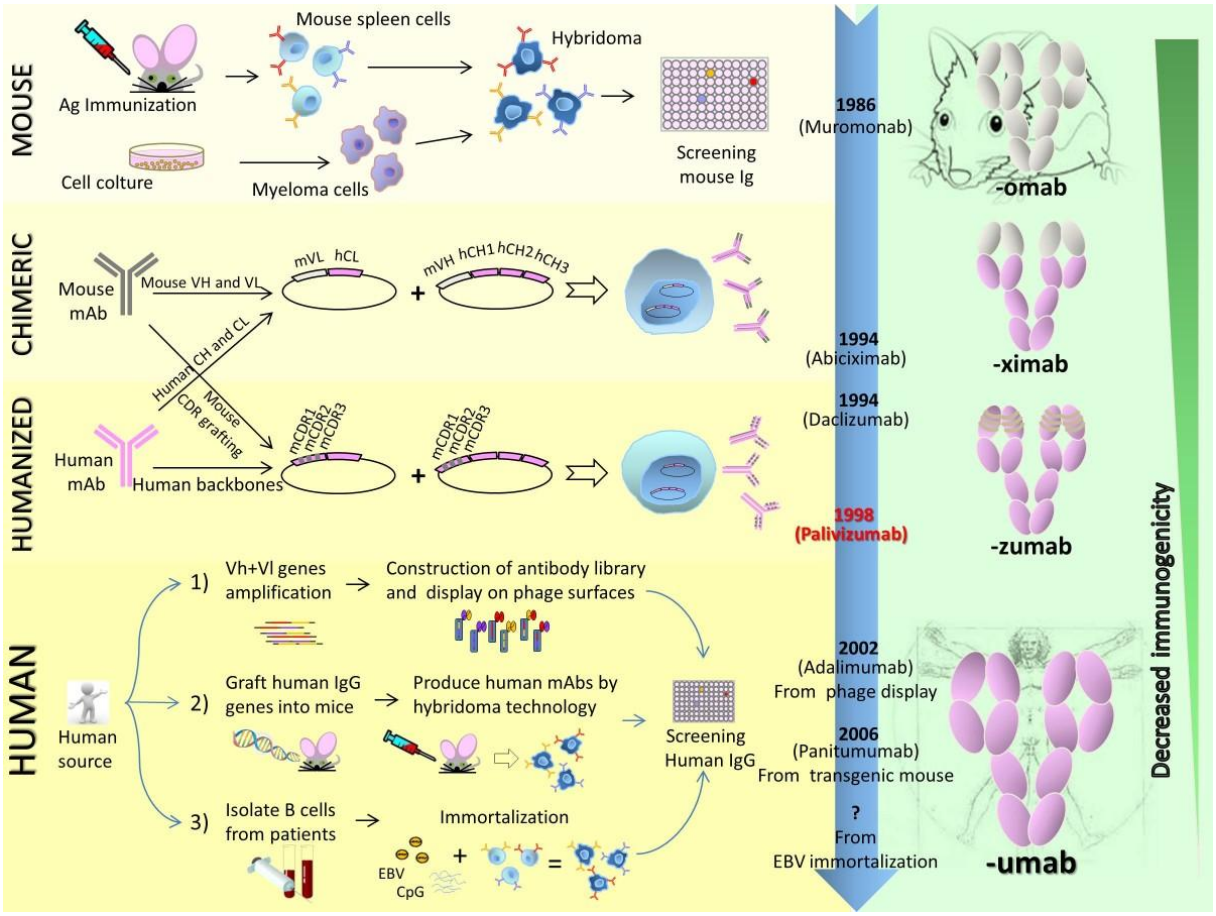

Figure 1.9: Schematic representation of strategies involved in generation of mouse, chimeric, humanized and fully human MA *(adapted from* Flego *et al.* [118]. Chimeric and humanized antibodies are composed of a human Ig scaffold with respective variable regions or CDRs from mouse origin (CDR grafting).
EBV: Epstein Barr Virus and CpG: CG dinucleotide.

1.3.2. Heavy-chain antibodies and nanobody

For a long time, Igs were supposed to be composed of four heteromeric polypeptide chains (two identical heavy chains and light chains each). However, in 1993 it was discovered that along with conventional Igs the members of the family Camelidae also express homomeric Igs consisting of only 2 polypeptide chains known as heavy-chain antibodies (HCAbs) [121] (Fig. 1.7). Depending on the species, about 10-80% of their IgG repertoire consists of HCAbs [112, 122, 123]. As their name advocates HCAbs is composed of only two heavy chains which also lack the C_H1 domain and thus have a lower molecular weight i.e. ~90 kDa (Fig. 1.7) [99]. Due to its small size (40 Å long X 24 Å wide) the variable fragment of a HCAbs i.e. V_{HH} is also referred as Vhh/nanobody (Nb). A nanobody is the smallest *in vivo* affinity-matured functional antigen-binding fragment (15 kDa) and can be expressed with very high yield in *E. coli* and yeast [110, 112].

1.3.2.1. Structure of nanobody

Amino acid sequence alignment analysis of V_{HH} and V_H revealed a high degree of sequence homology between the variable domains of a V_{HH} and a human V_H3 sub-family of Abs. Based on the DNA sequence homology, the V_H domains of Abs can be classified in six discrete sub-families i.e. V_H1- V_H6

[124]. V_H3 sub-family of Abs is the most abundant and highest thermodynamically stable human antibody in the natural repertoire. Only these Abs can be expressed as soluble protein with very high yield in bacterial systems [125]. However, there are still two noteworthy differences between Vhh and V_H3 sub-family (Fig. 1.8). Firstly, four amino acid residues (V^{42}, G^{49}, L^{50} and W^{52}) responsible for the interaction between V_L and V_H located in the framework region 2 (FR2) of V_H3 are substituted by hydrophilic residues (F^{42}, E^{49}, R^{50} and G^{52}) in V_{HH} which results into increased solubility and stability of Nbs. Secondly, the CDR1 and CDR3 (13-24 amino acid in Vhh compared to 9-12 and 9-17 amino acid in mouse and human V_H, respectively) domains of the Vhh are considerably longer than V_H [126]. The extended CDR3 is probably an evolutionary replacement for the V_L domain (antigen-binding surface). Furthermore, the elongated CDR3 domain is involved in the binding to the catalytic clefts and therefore has enzyme inhibitory properties. However, elongation of CDR3 loop also introduces high flexibility and thereby hampers its capability to bind to an antigen. A disulfide-bridge formed between CDR3 and CDR1 or FR2 stabilizes this flexibility and maintains its inhibitory function [106].

1.3.2.2. Production of nanobody

Like MA generation, Nb generation also requires animals (mammals) but only from the family of camelidae (typically alpaca or IIama). Generation of Nbs involve isolation of lymphocytes from the blood collected from immunized camelids, mRNA isolation from isolated lymphocytes, cDNA preparation and subsequent cloning of Vhh collection into a phage display vector, followed by selection of antigen specific Vhh by 2-4 rounds of panning [127]. Later the Vhh is cloned into expression vectors for production in large amounts [128-130].

1.3.2.3. Properties of nanobody

Usually, Nbs denature in 2.3–3.3 M guanidinium concentration at 60–80 °C [131]. However, Nbs are robust under stringent conditions (detergents and high concentrations of urea) and resist chemical and thermal denaturation [132]. Even after incubation for 1 week at 37 °C, Nbs are fully active (80-100%) [128] and also appear to be stable up to 70°C [133]. Like MA, Nbs also get denature and lose its activity upon incubation at 90 °C, but unlike MA, Nbs are renaturable [134]. The stability of a Nb can also be increased by introducing an extra disulfide bond by inserting Cys at position 54 and 78 [135, 136] which also resulted into generation of highly chymotrypsin- or pepsin-resistant Nbs [137]. Trypsin-resistant Nbs have also been obtained after random mutagenesis [138]. Generation of protease resistant Nbs has opened the new door for oral administration of Nbs.

The antigen-binding area of a nanobody is formed by the elongated CDR3 domain folded in a convex surface (Fig. 1.8) and most of the catalytic sites are harboured in a cleft. Therefore, Nbs are also efficient enzyme inhibitors [127, 139, 140]. Along with a direct inhibition of the enzyme, Nbs can inhibit an enzyme by allosteric inhibition as evident from the studies of Nbs with shorter CDR3 domain [141]. Where some Nbs inhibit enzyme activity, others can also activate enzymatic activity, possibly due to stabilization of enzyme in an open conformation [86, 90]. The small size, high stability, rapid clearance from blood and most important a high degree of sequence homology with human V_{H3} sequences (only 10 amino acids difference in the FR regions) reduces the risk for an immunological

reaction. Indeed, no immune response against the Nbs was detected in mice or humans injected with Nb-containing constructs [142-144]. This risk of immunogenicity can additionally be decreased by humanization of some residues without introducing any deleterious effect like loss of solubility, stability and yield etc. [145].

1.3.2.4. Use of nanobody

Characteristics like a renewable/sustainable source, high affinities, specificity similar to MA, the unique structure, their beneficial biochemical and economic properties (size, stability, solubility, expression levels, reversible folding, recognition of non-conventional epitopes and production cost) and high sequence identity with human V_H3 subfamily of antibodies encouraged researches and pharmaceutical and biotech companies to employ Nbs as a research tool, as diagnostics and for therapeutic applications. Moreover, in some cases, the large size of a conventional MA interferes with the access of hidden and essential epitopes on pathogenic agents, like bacteria, parasites and viruses. In such cases Nbs will have special advantages as therapeutics [112, 146].

To date no Nb has been approved for therapeutic purposes. However, promising results were obtained for several Nbs evaluated in clinical trials (Table 1.3) [147]. In 2012, Boehringer Ingelheim along with Ablynx has also submitted clinical trials application for Nanobody-based Alzheimer's treatment. In addition, Nbs are also actively being investigated against scorpion toxins, antibacterial toxins and antisnake venom [148, 149]. However, Clinical trial phase 1 of CXCR4 inhibiting Nb ALX-0651 as anti HIV agent for safety and effectiveness was terminated within one year [147]. So far no immunological response towards the nanobodies has been observed. To modify other desirable properties like plasma half-life and potency other nanobody engineering techniques like generation of bispecific or multivalent construct can be implemented. Generation of a bispecific construct towards a target molecule and serum albumin (ALX-0141) will extend the half-life [142] and multivalent constructs will increase the avidity of Nb leading to increase in its potency [149].

Table 1.3: Nanobodies in Clinical trial [147].

Nanobody	Target	Pathology	Administration	Clinical trial
ALX-0061	IL-6R	Rheumatoid arthritis	Intravenous	Phase-2
ALX-0081/ALX-0681 (Caplacizumab)	vWR	PCI and TTP	Intravenous	Phase-2
ATN-103 (Ozoralizumab)	TNFα	Rheumatoid arthritis	Intravenous	Phase-2
Vhh batch 203027	Rotavirus	Children diarrhea	Oral	Phase-2
ALX-0171	Human RSV	Children RSV infection	Intravenous	Phase-1
ALX-0141	RANKL	Bone metastasis, osteoporosis and rheumatoid arthritis	Intravenous	Phase-1

PCI; percutaneous coronary intervention, RSV; respiratory syncytial virus and TTP; thrombotic thrombocytopenic purpura.

1.4. Goal of study

- The first objective was to evaluate the physiological role of the zymogen activity of TAFI in the regulation of fibrinolysis (**Chapter 2**).

- The second objective was to identify and to characterize MA that impair the activation of TAFI through different working mechanisms (**Chapter 3**).

- The third objective was to study the relative contribution of the different TAFI activators regulating fibrinolysis (**Chapter 3 and Chapter 4**).

- The fourth objective was to confirm the inhibitory properties of the MA directed toward human TAFI in *in vivo* and *in vitro* models using hTAFI transgenic mice (**Chapter 5**).

CHAPTER 2

Increased Zymogen Activity Of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis

Niraj Mishra, Karlien Buelens, Stéphanie Theyskens,

Griet Compernolle, Ann Gils and Paul J. Declerck

Laboratory for Therapeutic and Diagnostic Antibodies,

Faculty of Pharmaceutical Sciences,

Katholieke Universiteit Leuven, Leuven

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2.1. Abstract

Background: Thrombin Activatable Fibrinolysis Inhibitor (TAFI) is a zymogen that can be activated by proteolytic cleavage into the active enzyme TAFIa. Hydrolysis of the C-terminal lysines on fibrin by TAFIa results in a down regulation of fibrinolysis. Recent studies demonstrated that the zymogen also exerts an intrinsic enzymatic activity.

Objectives: Our objective was to identify and characterize zymogen-stimulatory nanobodies.

Methods and results: Screening of 24 nanobodies against TAFI revealed that two nanobodies (*i.e.* Vhh-TAFI-a51 and Vhh-TAFI-i103) were able to stimulate the zymogen activity 10- to 21-fold compared to the baseline zymogen activity of TAFI. The increase in catalytic efficiency can be attributed mainly to an increased catalytic rate, since no change in K_M-value was observed. The stability, the susceptibility towards PTCI and GEMSA and the kinetics of the stimulated zymogen activity differ significantly from those of TAFIa activity. Epitope mapping revealed that both Asp^{75} and Thr^{301} are major determinants in the binding of these nanobodies to TAFI. Localization of the epitope strongly suggests that this instability is due to a disruption of the stabilizing interactions between the activation peptide and the dynamic flap region (residues 296-350).

In TAFI-depleted plasma reconstituted with a non-activatable variant of TAFI (TAFI-R92A), clot lysis could be prolonged by nanobody-induced stimulation of its zymogen activity as well as by increasing its concentration.

Conclusions: Increasing the zymogen activity of TAFI results in an antifibrinolytic effect.

2.2. Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) is a 56 kDa zymogen secreted by the liver. It circulates in the blood at concentrations between 75 and 275 nmol/L [30, 31]. TAFI can be activated by thrombin, thrombin/thrombomodulin or plasmin, resulting in the activation peptide (20 kDa) and the TAFIa moiety (36 kDa) [20, 21, 38]. TAFIa is a carboxypeptidase and attenuates fibrinolysis by removing C-terminal lysines from partially degraded fibrin. This results in a down-regulation of tissue-type plasminogen activator (t-PA) mediated plasmin generation. There are no known physiological inhibitors of TAFIa. Nevertheless, its activity is regulated by the thermal instability of TAFIa and depends on the TAFI isoform. The 147Ala/Thr polymorphism does not affect TAFIa instability, in contrast to the 325Thr/Ile polymorphism. The isoforms i.e. TAFIa-Ala147-Thr325 (TAFIa-AT) and TAFIa-Thr147-Thr325 (TAFIa-TT) have a half-life of 8 minutes whereas TAFIa-Ala147-Ile325 (TAFIa-AI) and TAFIa-Thr147-Ile325 (TAFIa-TI) have a half-life of 15 minutes at 37°C [49]. The mechanism of this instability has been revealed by solving the crystal structure of TAFI [55]. An interaction between the activation peptide and the dynamic flap region (Phe^{296} to Trp^{350}) in the TAFIa moiety stabilizes the conformation of TAFI. Upon activation of TAFI, the activation peptide is released, resulting in an increased mobility of the flap region. Consequently, TAFIa is inactivated due to conformational changes of the catalytic site. A few years ago, a TAFI variant TAFI-A147-C305-I325-I329-Y333-Q335 (TAFI-ACIIYQ) was generated with a 180-fold longer half-life [53]. These mutations increase the interactions within the dynamic flap region and between the flap region and the stable core of the TAFIa moiety [151], thereby resulting in a reduced rate of conformational inactivation. The TAFIa stability is one of the crucial factors that influence the threshold dependent regulation of fibrinolysis. TAFIa inhibits fibrinolysis when its concentration is above a certain threshold which depends on the t-PA concentration [64, 65].

Recently, it was reported that not only TAFIa, but also the zymogen itself exerts a carboxypeptidase activity [66, 152]. The TAFI zymogen has a 41-fold decreased catalytic rate in comparison with TAFIa. This is partially compensated by a 2-fold increased affinity for the substrate, resulting in an 18-fold lower catalytic efficiency [66]. There is still some controversy whether the zymogen activity of TAFI has a physiological role in the regulation of fibrinolysis. Valnickova *et al.* described that the down regulation of fibrinolysis by TAFI in a clot lysis assay without addition of exogenous thrombomodulin must be due to the zymogen activity of TAFI since nearly no TAFIa is generated under these conditions [66]. However, results of a study by Willemse *et al.* suggest that there was TAFIa generated under these circumstances [68]. In another study, Foley *et al.* suggested that the zymogen activity cannot attenuate fibrinolysis, since TAFI zymogen is unable to cleave fibrin degradation products [40].

Recently, we described the generation of a panel of nanobodies directed towards TAFI and identified nanobodies that inhibit TAFI(a) [90]. Surprisingly, among the initially generated panel, two nanobodies appeared to stimulate significantly the zymogen activity. In this study we report the characterization of these nanobodies, the properties of the stimulated zymogen activity and the effect of an increased zymogen activity on clot lysis.

2.3. Materials and methods

2.3.1. Materials

Human thrombin, human plasmin and rabbit thrombomodulin were purchased from Sigma-Aldrich, Enzyme Research Labs (South Bend, UK) and American Diagnostica (Greenwich, CT, USA), respectively. H-D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), aprotinin, hippuryl-L-arginine, guanidinoethyl-mercaptosuccinic acid (GEMSA) and potato tuber carboxypeptidase inhibitor (PTCI) were obtained from Biomol Research Labs (Plymouth Meeting, PA, USA), Fluka (Buchs, Switzerland), Bachem (Bubendorf, Switzerland) and Calbiochem (La Jolla, CA, USA), respectively. Tissue-type plasminogen activator (Actilyse®) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Citrated plasma of 33 healthy individuals was collected in-house with their written consent and was pooled for clot lysis experiments.

2.3.2. Methods

2.3.2.1. Generation and purification TAFI

Wild-type recombinant human TAFI-Thr^{147}-Ile^{325} (TAFI-TI) and TAFI-ACIIYQ were produced in HEK293(T) cells and purified from the conditioned medium at 4 °C by affinity chromatography using monoclonal antibodies MA-T27G4 and MA-T4E3, respectively. All other TAFI variants were constructed and purified in a similar way, using MA-T27G4, as described before [88]. One mutant, TAFI-TI-R92A, in which Arg^{92} was replaced by Ala, was specifically designed to obtain a non-activatable TAFI variant.

2.3.2.2. Generation of nanobodies

The generation of the nanobody library as well as the selection procedure through panning on TAFI-ACIIYQ either in its intact form or in its active form, was described previously [90]. Screening of the anti-TAFI nanobodies (n = 24) for a stimulatory effect on TAFI zymogen activity resulted in the identification of the nanobodies Vhh-TAFI-a51 and Vhh-TAFI-i103.

2.3.2.3. Evaluation of the effect of the nanobodies on the catalytic activity of the TAFI zymogen

TAFI-TI or TAFI-TI-R92A (concentration during substrate conversion: 40 nmol/L) was incubated with nanobody (at a concentration ranging between 1 and 32-fold molar ratio over TAFI) for 10 min at 37 °C, prior to incubation with Hippuryl-Arg (Hip-Arg, concentration during substrate conversion: 11.1 mmol/L). Substrate conversion was allowed for 1 hour at 37 °C. Reactions were stopped and the absorbance was measured as described before [90]. To be able to express the TAFI activity in U/mg, a standard spanning 15.6 µmol/L to 2 mmol /L hippurate was used to quantify the amount of hippurate formed by TAFI [153]. One unit (U) carboxypeptidase activity is defined as the amount of enzyme converting 1 micromole of substrate per minute at 37 °C.

2.3.2.4. Stability of the stimulated zymogen activity

TAFI-TI or TAFI-TI-R92A (concentration during substrate conversion: 40 nmol/L) was incubated in the absence or the presence of nanobody (16-fold molar excess over TAFI) at different time intervals ranging from 0 to 120 minutes at 37 °C. Substrate conversion was allowed for 1 hour at 37 °C and the activity was determined as described above.

2.3.2.5. Evaluation of the effect of the nanobodies on TAFI fragmentation

To exclude that the observed increase in activity was due to a proteolytic cleavage induced by the nanobodies, TAFI-TI (800 nmol/L) was incubated with nanobodies (32-fold molar excess over TAFI) at 37 °C for 10 minutes. Sodium dodecyl sulfate (1% final concentration) was added, samples were heated for 30 seconds at 100 °C and subsequently analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using PhastGel™ gradient 10-15% gels, followed by silver staining.

2.3.2.6. Inhibitory effect of PTCI on the (stimulated) zymogen activity of TAFI-TI and TAFI-TI-R92A

TAFI-TI (concentration during substrate conversion: 40 nmol/L) or TAFI-TI-R92A (concentration during substrate conversion: 20 nmol/L) was incubated either with buffer or with nanobody (32-fold molar excess over TAFI) for 10 min at 37°C. Subsequently, either buffer or PTCI (concentration during substrate conversion, ranging between 0.11 and 56 µmol/L) was added and incubated for 10 min at 37 °C. After addition of Hip-Arg (concentration during substrate conversion: 11.1 mmol/L), substrate conversion was allowed for 1 hour at 37 °C. From the comparison of the stimulated zymogen activity in the absence and presence of PTCI, the extent of inhibition of zymogen activity was calculated in U/mg and expressed as percentage inhibition.

2.3.2.7. Inhibitory effect of GEMSA on the (stimulated) zymogen activity of TAFI-TI and TAFI-TI-R92A

TAFI-TI (concentration during substrate conversion: 40 nmol/L) or TAFI-TI-R92A (concentration during substrate conversion: 20 nmol/L) was incubated either with buffer or with nanobody (32-fold molar excess over TAFI) for 10 min at 37°C. Subsequently, either buffer or GEMSA (concentration during substrate conversion: ranging between 1.4 and 90 µmol/L) was added and incubated for 10 min at 37 °C. After addition of Hip-Arg (concentration during substrate conversion: 11.1 mmol/L), substrate conversion was allowed for 1 hour at 37 °C. From the comparison of the stimulated zymogen activity in the absence and presence of GEMSA, the extent of inhibition of zymogen activity was calculated in U/mg and expressed as percentage inhibition.

2.3.2.8. The effect of nanobodies on the enzymatic properties of the TAFI zymogen

Hydrolysis of Hip-Arg by TAFI-TI was determined in the presence and absence of nanobody. TAFI (concentration during substrate conversion: 275 nmol/L) was incubated with nanobody (32-fold molar excess over TAFI) for 10 minutes at 25 °C. In the control condition, without addition of nanobody, a

higher concentration TAFI-TI was used (*i.e.* 936 nmol/L) in order to obtain measurable substrate conversion rates. Subsequently, Hip-Arg was added (concentrations during substrate conversion: 0.25 – 6 mmol/L for the stimulated zymogen and 0.125 – 4 mmol/L for the control without stimulating nanobody) and the rate of Hip-Arg hydrolysis was monitored at 254 nm for 10 min at 25 °C. The k_{cat} and K_M values were determined by nonlinear regression of the data to the Michaelis-Menten equation [90].

2.3.2.9. Evaluation of the effect of zymogen activity and of nanobody-stimulated zymogen activity on *in vitro* clot lysis

To evaluate the mere effect of the zymogen activity on clot lysis the generation of TAFIa must be ruled out. Therefore clot lysis experiments were designed using TAFI-depleted plasma reconstituted with the non-activatable variant TAFI-TI-R92A. Alternatively in control experiments TAFI-TI was added. TAFI-depleted plasma was obtained using MA-T4E3 coupled Sepharose 4B as described earlier [154]. Clot lysis was performed in a 96-well microtiter plate as described previously [154] with some modifications. Pooled TAFI-depleted plasma (final concentration: 30%) was mixed either with dilution buffer (10 mM Tris, 0.01%tween 20; pH 7.5) or with TAFI variants (final concentration: 54 nM), and/or nanobodies (final concentration: 1728 nM, resulting in a 32-fold molar ratio over TAFI). The plate was incubated at 37°C for 10 min and t-PA (120 pM) was added. Then clot formation was induced by addition of $CaCl_2$ (10.6 mM) and the plate was read at 2 min intervals at 405 nm at 37°C. The 50% clot lysis time was determined and defined as the time interval between the time point of full clot formation to the midpoint of the maximum turbidity to clear transition. The effect of added compounds (i.e. TAFI variant and/or nanobody) on clot lysis time was expressed relative to the clot lysis time of TAFI depleted plasma (54.5 ± 17 min). To further explore the putative effect of zymogen activity of TAFI-TI-R92A on clot lysis, clot lysis experiments were also carried out using a five-fold higher concentration of this variant (final concentration: 270 nM).

2.3.2.10. Affinity measurements

Affinity constants for the binding between the nanobodies and TAFI-TI, either in its proenzyme form or in its activated form, were determined using the Biacore 3000 analytical system (Biacore AB, Uppsala, Sweden) equipped with a CM-5 sensor chip [90]. Nanobodies were covalently coupled to approximately 1200 resonance units using a concentration of 5 µg/mL nanobody in acetate buffer (10 mmol/L; pH 4.5). TAFI-TI (either intact or activated) was diluted in HBS-EP buffer (Biacore) to concentrations between 5 and 200 nmol/L and injected at a flow rate of 30 µL/min. After each cycle, the chip was regenerated with 10 µL of 10 mmol/L glycine; pH 1.5. Analysis of the association and dissociation phases was performed using the Biacore 3000 software (Langmuir binding, local fit). All experiments were performed at least three times independently. Activated TAFI-TI was prepared as follows. TAFI-TI (final concentration: 804 nmol/L in Tris buffer: 20 mmol/L, 100 nmol/L NaCl; pH 7.4) was incubated for 10 minutes at 37 °C with $CaCl_2$, thrombin and thrombomodulin (concentration at activation step: 5 mmol/L, 20 nmol/L and 5 nmol/L, respectively). The activation was stopped by addition of PPACK (final concentration: 30 µmol/L).

2.3.2.11. Statistical data analysis

Quantitative data were summarized by mean and standard deviation obtained from at least 3 independent experiments. Statistical significance was determined with the unpaired *t*-test using Graph Pad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). Values were considered as statistically significant at two-tailed p-values smaller than 0.05. For the clot lysis experiments paired, two-tailed *t*-test was performed.

2.4. Results

2.4.1. Stimulation of the zymogen activity

The zymogen activity of TAFI-TI without incubation with nanobody was 0.12 ± 0.04 U/mg which is in the range of activities reported before (Table 2.1) [152, 153]. To exclude the generation of TAFIa activity in clot lysis experiments (see below) a non-activatable TAFI mutant (TAFI-TI-R92A) was generated. The activatability of this variant was 100-fold lower compared to that of TAFI-TI. The zymogen activity of TAFI-TI-R92A, 0.63 ± 0.11 U/mg, was 5-fold higher ($p < 0.0001$ *versus* TAFI-TI).

Table 2.1: Effect of Nbs on zymogen activity of TAFI variants.

	Zymogen activity of TAFI		
	No addition	TAFI-Vhh-a51	TAFI-Vhh-i103
TAFI-TI (wt)	0.12 ± 0.04	2.07 ± 0.23 (17)	1.15 ± 0.15 (10)
TAFI-ACIIYQ	0.17 ± 0.14 [1.4]	3.20 ± 0.79 (19)	2.89 ± 1.01 (17)
TAFI-TI-R92A	0.63 ± 0.11 [5]	13.3 ± 0.84 (21)	8.92 ± 0.28 (14)

TAFI-ACIIYQ: stable human TAFI variant; values presented in [] represent fold change in zymogen activity of TAFI variant compared to TAFI-TI and values presented in () represent fold change in zymogen activity of TAFI variant in presence of Nb compared to no addition.

When TAFI-TI was incubated with either Vhh-TAFI-a51 or Vhh-TAFI-i103 at a 32-fold molar ratio of nanobody over TAFI, the zymogen activity was stimulated significantly, resulting in an activity of 2.07 ± 0.23 U/mg and 1.15 ± 0.15 U/mg, respectively (Table 2.1). For TAFI-TI-R92A, a stimulation of zymogen activity to 13.3 ± 0.84 U/mg and 8.92 ± 0.28 U/mg, respectively, was observed. Dose-response curves reveal that the stimulatory effect of the nanobodies is dose-dependent (Fig. 2.1). The zymogen activity of the stable variant TAFI-AI-CIYQ is also stimulated by these nanobodies (0.17 ± 0.14 U/mg in the absence *versus* 3.20 ± 0.79 U/mg and 2.89 ± 1.01 U/mg, in the presence of a 32-fold molar excess of Vhh-TAFI-a51 or Vhh-TAFI-i103, respectively).

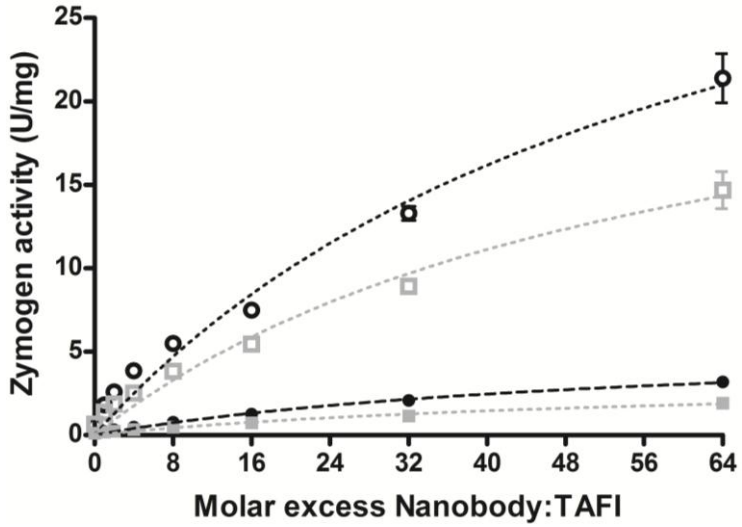

Figure 2.1: Dose-dependent effect of nanobodies on zymogen activity of TAFI variants. Dose-dependent effect of Vhh-TAFI-a51 (circles) and Vhh-TAFI-i103 (squares) on the zymogen activity of TAFI-TI (closed symbols) and TAFI-TI-R92A (open symbols). (Mean ± SD, n ≥ 3).

2.4.2. Effect of the nanobodies on TAFI fragmentation

To exclude the generation of TAFIa activity due to activation by cleavage of TAFI-TI by the nanobodies, TAFI-TI was analyzed by SDS-PAGE in the absence and presence of Vhh-TAFI-a51 and Vhh-TAFI-i103. Only two bands were observed: one band corresponding to intact TAFI (56 kDa) and one band corresponding to nanobody (15 kDa). The typical 36 kDa-fragment, generated upon activation of TAFI, could not be observed (data not shown); indicating that incubation with the nanobodies does not result in an activation of TAFI to TAFIa.

2.4.3. Stability of the (stimulated) zymogen activity

In order to determine the stability of the zymogen activity, TAFI-TI and TAFI-TI-R92A zymogen activity was analyzed at different time points in the absence and in the presence of the nanobodies at 37 °C. Because of the low activity of unstimulated TAFI-TI zymogen it appeared impossible to evaluate the half-life. For zymogen activity of TAFI-TI-R92A a half-life of 19 ± 3.2 minutes was found. The stimulated TAFI-TI zymogen was unstable at 37 °C with half-lives of 43 ± 10 minutes and 32 ± 4.0 minutes, for Vhh-TAFI-a51 and Vhh-TAFI-i103, respectively. Similar values were obtained for the stimulated zymogen activity of TAFI-TI-R92A, i.e. half-lives of 35 ± 3.1 and 36 ± 4.6 minutes, respectively. Evaluation of the stimulated zymogen activity of the stable TAFI variant (TAFI-ACIIYQ) in the presence of Vhh-TAFI-a51 and Vhh-TAFI-i103 revealed a residual activity of 85 ± 13 % and 95 ± 6 %, respectively after 120 minutes at 37 °C, indicative for half-lives exceeding much more than 120 minutes.

2.4.4. Inhibitory effect of PTCI on the (stimulated) zymogen activity

Because of the low activity of unstimulated TAFI-TI zymogen, its inhibition by PTCI could not be evaluated reliably. Addition of PTCI to TAFI-TI-R92A resulted in a dose-dependent inhibition of the zymogen activity with an IC_{50}-value of 3.0 ± 0.53 µmol/L.

Addition of PTCI after stimulation of the zymogen activity of TAFI-TI by Vhh-TAFI-a51 and Vhh-TAFI-i103 resulted in dose-dependent inhibition of the zymogen activity with IC_{50} values of 15 ± 3.9 µmol/L and 17 ± 3.5 µmol/L, respectively (Fig. 2.2A). Corresponding IC_{50} values for inhibition of stimulated zymogen activity of TAFI-TI-R92A were 0.98 ± 0.05 and 1.9 ± 0.16 µmol/L, respectively. IC_{50} values for inhibition of activated TAFI by PTCI were 0.22 ± 0.02 µmol/L, and were not affected by the nanobodies.

2.4.5. Inhibitory effect of GEMSA on the (stimulated) zymogen activity

Because of the low activity of unstimulated TAFI-TI zymogen its inhibition by GEMSA could not be evaluated reliably. Addition of GEMSA to TAFI-TI-R92A resulted in a dose-dependent inhibition of the zymogen activity with an IC_{50}-value of 8.1 ± 0.84 µmol/L.

Addition of GEMSA after stimulation of the zymogen activity of TAFI-TI by Vhh-TAFI-a51 and Vhh-TAFI-i103, resulted in dose-dependent inhibition of the zymogen activity with IC_{50} values of 5.8 ± 0.46 µmol/L and 6.6 ± 0.67 µmol/L, respectively (Fig. 2.2B). Corresponding IC_{50} values for inhibition of stimulated zymogen activity of TAFI-TI-R92A were 15 ± 0.99 and 13 ± 1.0 µmol/L, respectively. IC_{50} values for inhibition of activated TAFI by GEMSA were 283 ± 34 µmol/L and were not affected by the nanobodies.

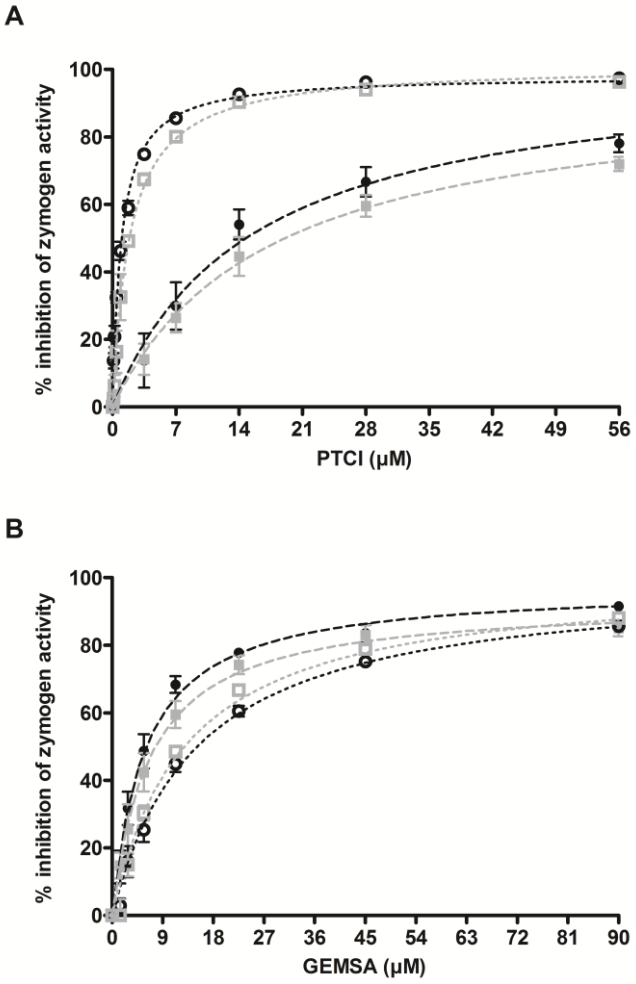

Figure 2.2: Effect of PTCI (A) and GEMSA (B) on stimulated zymogen activity of TAFI variants. Dose-response curve of inhibition by PTCI or GEMSA of the zymogen activity of TAFI-TI (closed symbols) and TAFI-TI-R92A (open symbols) stimulated by Vhh-TAFI-a51 (circles) and Vhh-TAFI-i103 (squares).(Mean ± SD, n ≥3).

2.4.6. Effect of the nanobodies on the enzymatic properties of TAFI

Addition of Vhh-TAFI-a51 and Vhh-TAFI-i103 resulted in a significant increase of the catalytic rate (k_{cat}) of Hip-Arg hydrolysis (Table 2.2). No effect was observed on K_M-values. Subsequently, the overall catalytic efficiency (k_{cat}/K_M) of the stimulated zymogen is significantly increased. The catalytic rate and catalytic efficiency of the zymogen stimulated by Vhh-TAFI-a51 differ significantly from those observed in the presence of Vhh-TAFI-i103 ($p < 0.05$ and $p < 0.0001$, respectively). Comparison with the enzymatic properties of TAFIa reveals that the K_M-values of the (stimulated) zymogen activity do

not differ significantly from the K_M-values of the TAFIa activity [90] . In contrast, the catalytic rate and the catalytic efficiency of TAFIa are significantly ($p < 0.0005$) higher than those observed for the (stimulated) zymogen activity.

Table 2.2: Enzyme kinetics of Hip-Arg hydrolysis by TAFI in the absence and presence of Vhh-TAFI-a51 and Vhh-TAFI-i103

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
No addition	1.21 ± 0.69	0.175 ± 0.063	0.240 ± 0.257
Vhh-TAFI-a51	1.19 ± 0.21	3.01 ± 0.32**	2.56 ± 0.19**
Vhh-TAFI-i103	1.72 ± 0.60	1.93 ± 0.10**	1.13 ± 0.31*
TAFIa†	1.21 ± 0.26	12.4 ± 2.04**	10.6 ± 1.90**

Mean ± SD, n ≥ 3. * $p < 0.001$ *vs.* 'no addition', ** $p < 0.0005$ *vs.* 'no addition'. †Data taken from [90].

Table 2.3: Effect of TAFI variants and zymogen-stimulatory nanobodies on clot lysis time in TAFI-depleted plasma

addition	Relative clot lysis time (1)	p-value	
none	1		
Vhh-TAFI-a51	0,96 ± 0,08		
Vhh-TAFI-i103	0,98 ± 0,04		
TAFI-TI	5,01 ± 0,35	**	
TAFI-TI + Vhh-TAFI-a51	4,14 ± 0,51	**	†
TAFI-TI + Vhh-TAFI-i103	4,41 ± 0,44	**	†
TAFI-TI-R92A	1,04 ± 0,07		
TAFI-TI-R92A + Vhh-TAFI-a51	1,63 ± 0,27	*	#
TAFI-TI-R92A + Vhh-TAFI-i103	1,54 ± 0,27	*	#
TAFI-TI-R92A (5-fold) (2)	1,23 ± 0,06	**	##

(1) Expressed as ratio of clot lysis time in the absence of any compound over clot lysis time in the presence of the indicated compound (see methods); Lysis time in the absence of any compound added was 55 ± 17 minutes;
(2) Evaluated at five-fold higher concentration;
Mean ± SD, n ≥ 3; * $p < 0.05$ *vs.* none, ** $p < 0.002$ *vs.* 'none', † $p < 0.01$ vs. TAFI-TI, # $p < 0.05$ vs. TAFI-TI-R92A, ## $p < 0.001$ vs. TAFI-TI-R92A.

2.4.7. Evaluation of the effect of zymogen stimulation nanobodies on *in vitro* clot lysis

Addition of the nanobodies to TAFI depleted plasma did not affect clot lysis times (Table 2.3). As expected addition of TAFI-TI significantly prolonged clot lysis times. Addition of the zymogen stimulatory nanobodies under these conditions slightly reduced (12 to 17 %) clot lysis times. As expected addition of the non-activatable variant TAFI-TI-R92A did not affect clot lysis under the standard conditions. However the zymogen stimulatory nanobodies significantly increased (50 to 60 %) clot lysis times in the presence of TAFI-TI-R92A. Addition of TAFI-TI-R92A at a five-fold higher concentration also resulted in a significant increase (20 %) of the clot lysis time (Table 2.3). It was verified using an ELISA specific for the released activation peptide of TAFI [155], that TAFI-TI-R92A was not activated under any of those conditions (data not shown).

2.4.8. Epitope mapping

Initially, all purified TAFI variants (n = 15) available in the lab were screened for their susceptibility towards stimulation by Vhh-TAFI-a51 or Vhh-TAFI-i103. Mutation of the following residues did not affect stimulation by neither of the two nanobodies: Val41, Gln45, Gly66, Ser70, Gln81, Asp87, Thr88, Ala95, Phe113, Arg117, His126, Glu132, Lys133, Ala148, Ile158, Tyr223, Ser305, Thr329, His333, His335 (data not shown). These residues are depicted in Fig. 2.3 (orange spheres).

Strikingly, the zymogen activity of both TAFI-TI-D75N and TAFI-AI-S305C-T329I-H333Y-H335Q-T301A (TAFI-AI-CIYQ-T301A) could not be stimulated by Vhh-TAFI-a51 and Vhh-TAFI-i103. Dose-response curves were performed and revealed that even at 64-fold molar excess of nanobody over TAFI no stimulatory effect could be observed. Both TAFI variants exerted a zymogen activity comparable to that of TAFI-TI and TAFI-AI-CIYQ (data not shown).

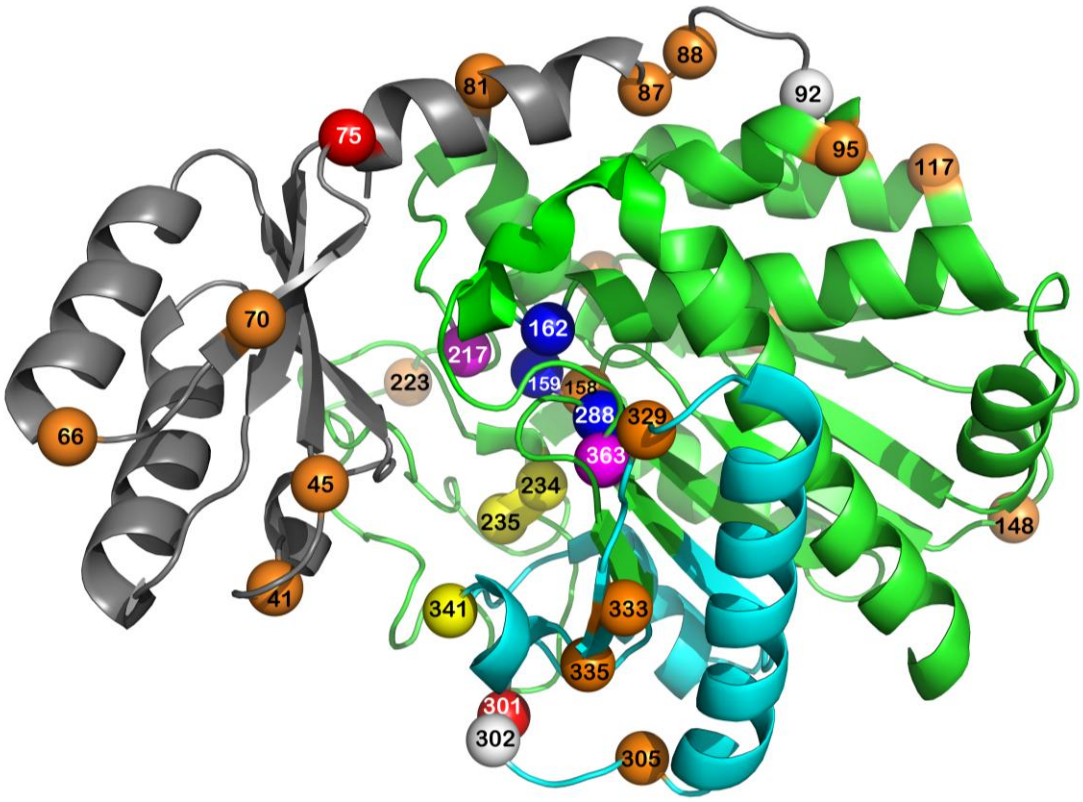

Figure 2.3: Structure of TAFI and localization of screened residues. Ribbon drawing of TAFI based on the structure of Marx *et al.* with the activation peptide shown in grey, the catalytic domain in green and the dynamic flap region (residues 296-350) in cyan [55]. Residues involved in hydrolysis, substrate binding and zinc binding are shown in magenta, yellow and blue spheres, respectively. The two thrombin cleavage sites are shown as white spheres. The residues for which a mutation did not affect the stimulatory effect of the nanobodies are depicted as orange spheres. The residues Asp^{75} and Thr^{301} for which mutation resulted in a loss of stimulatory effect are depicted as red spheres.

The binding of Vhh-TAFI-a51 and Vhh-TAFI-i103 to TAFI variants was analyzed by surface plasmon resonance (Table 2.4). A weak binding was observed for intact TAFI-TI as well as for intact TAFI-AI-CIYQ with K_A-values of 4.5 and 3.8 x 10^6 M^{-1}, respectively. Upon activation of TAFI, a 10-fold higher affinity was observed, mainly due to a 10-fold higher association rate (k_a). In line with the

absence of any functional effect, no binding could be detected between the nanobodies and TAFI-TI-D75N or TAFI-AI-CIYQ-T301A, neither before nor after activation.

Taken together, these data suggest that both Asp^{75} and Thr^{301} (Fig. 2.3, red spheres) play a role in the binding of the zymogen stimulatory nanobodies to TAFI

Table 2.4: Binding parameters for Vhh-TAFI-a51 and Vhh-TAFI-i103. k_a, k_d and K_A were determined for binding of the nanobodies to the different TAFI variants using surface plasmon resonance.

	Vhh-TAFI-i103			Vhh-TAFI-a51		
	K_A (M^{-1})	k_d (s^{-1})	k_a (M^{-1} s^{-1})	K_A (M^{-1})	k_d (s^{-1})	k_a (M^{-1} s^{-1})
TAFI-TI	$3.83 \pm 0.64 \times 10^{6}$	$2.35 \pm 0.29 \times 10^{-3}$	$9.05 \pm 1.89 \times 10^{3}$	$4.52 \pm 1.07 \times 10^{6}$	$2.53 \pm 0.21 \times 10^{-3}$	$1.13 \pm 0.23 \times 10^{4}$
TAFIa-TI	$3.72 \pm 2.04 \times 10^{7}$	$6.26 \pm 0.61 \times 10^{-3}$	$2.30 \pm 1.23 \times 10^{5}$	$6.01 \pm 3.21 \times 10^{7}$	$5.14 \pm 0.54 \times 10^{-3}$	$3.97 \pm 1.37 \times 10^{5}$
TAFI-TI-D75N	NB	NB	NB	NB	NB	NB
TAFIa-TI-D75N	NB	NB	NB	NB	NB	NB
TAFI-AI-CIYQ	$2.29 \pm 1.00 \times 10^{6}$	$4.87 \pm 0.33 \times 10^{-3}$	$1.12 \pm 0.51 \times 10^{4}$	$3.79 \pm 1.46 \times 10^{6}$	$3.34 \pm 0.36 \times 10^{-3}$	$1.25 \pm 0.44 \times 10^{4}$
TAFIa-AI-CIYQ	$6.08 \pm 1.99 \times 10^{7}$	$6.28 \pm 0.35 \times 10^{-3}$	$3.77 \pm 1.06 \times 10^{5}$	$9.48 \pm 3.83 \times 10^{7}$	$4.81 \pm 0.51 \times 10^{-3}$	$4.41 \pm 1.32 \times 10^{5}$
TAFI-AI-CIYQ-T301A	NB	NB	NB	NB	NB	NB
TAFIa-AI-CIYQ-T301A	NB	NB	NB	NB	NB	NB

Mean ± SD, n ≥ 3. No binding (NB), $K_A \leq 10^{6}$ M^{-1}

2.5. Discussion

Until recently it was believed that TAFI needs to be activated in order to exert a carboxypeptidase activity. The TAFIa moiety is thermally unstable with a half-life between 8 and 15 minutes at 37°C [49]. It is generally accepted that this instability contributes to the regulation of the antifibrinolytic potential of TAFI. A few years ago, it was reported that also the TAFI zymogen exerts some carboxypeptidase activity. However, the physiological relevance of this activity remained a matter of debate [40, 66-68]. The present study demonstrates that the zymogen activity can be modulated to accept larger substrates, including C-terminal lysines on partially degraded fibrin and eventually resulting in an effect on clot lysis.

During our efforts to look for agents that may affect TAFI or TAFIa properties, we identified two nanobodies that were able to stimulate the zymogen activity. To the best of our knowledge stimulation of the zymogen activity of TAFI by any agent has not been described yet. The identified nanobodies increase the zymogen activity of TAFI-TI as well as that of the non-activatable TAFI-TI-R92A variant 10- to 21-fold compared to their respective baseline zymogen activity. However, for TAFI-TI the stimulated zymogen activity is still about 10 to 30-fold lower than the TAFIa activity, generated upon proteolytic activation of TAFI, which is about 18 to 31 U/mg [53, 153]. In contrast, for TAFI-TI-R92A, exhibiting a higher zymogen activity, the stimulated zymogen activity reaches values up to 13 U/mg, only 1.4 to 2.4-fold lower than the typical activity of activated TAFI.

Absence of any fragmentation product upon incubation of TAFI with these nanobodies and a stimulatory effect on the non-activatable variant TAFI-TI-R92A demonstrates that the observed increase in activity can be solely attributed to an increased zymogen activity and not to the formation of TAFIa. Moreover, the properties of the stimulated zymogen activity (*i.e.* stability, susceptibility towards PTCI and GEMSA and kinetics) differ significantly from the properties of TAFIa activity.

Indeed, the half-life of the stimulated zymogen activity is about 2-fold longer than the half-life of TAFIa at 37°C (30-40 minutes *vs.* 15 min) [49]. However, since the instability of the stimulated zymogen is also temperature dependent (i.e. the instability was much less pronounced at 25 °C, data not shown) and the stimulated zymogen activity of the stable variant TAFI-ACIIYQ also shows a significantly prolonged half-life compared to that of the stimulated zymogen activity of TAFI, there are some similarities between the stability of the stimulated zymogen and the stability of TAFIa.

Another difference between the stimulated zymogen activity and the TAFIa activity is the susceptibility towards the inhibitors PTCI and GEMSA. PTCI inhibits the stimulated zymogen activity with an IC_{50} in the micromolar range, in contrast to the IC_{50} for TAFIa which is in the nanomolar range [99]. Even though Valnickova *et al.* reported an inhibitory effect of PTCI on the zymogen activity [66, 156], a study by Foley *et al.* demonstrated that the zymogen activity of TAFI is not inhibited by PTCI [40]. The inhibition of the stimulated zymogen activity by PTCI further indicates that the active-site pocket in the nanobody-stimulated TAFI is more readily accessible than the active-site pocket in the non-stimulated zymogen. On the other hand the data obtained with GEMSA confirms the accessibility of the active-site pocket, but surprisingly, the IC_{50}-values for inhibition of the (stimulated) zymogen

activity are even 20- to 50-fold lower compared to the IC_{50}-values for inhibition of TAFIa. This confirms that the characteristics of the stimulated zymogen activity are clearly distinct from those of the TAFIa activity and indicates that the affinity of GEMSA for binding into the active-site pocket is higher in the zymogen forms (either non-stimulated, e.g. TAFI-TI-R92A, or stimulated) than in the activated form.

Also the results on the kinetics indicate that the characteristics of the stimulated zymogen activity differ from those of the TAFIa activity. The catalytic rate and the catalytic efficiency of the stimulated zymogen activity are about 10-fold increased compared to the zymogen activity, however, the catalytic rate and catalytic efficiency are still 5- to 10-fold lower compared to those of the TAFIa activity. It is important to note that the K_M-values of the substrate Hip-Arg as determined towards the stimulated zymogen, the zymogen and the activated TAFI are not significantly different. This indicates that the binding of the substrate Hip-Arg to the stimulated zymogen is not improved compared to its binding to the zymogen. In contrast, the observed increase in catalytic efficiency reflects an accelerated hydrolysis of Hip-Arg, which is suggestive for an induced conformational change of the catalytic site, consequent to the binding of the nanobodies.

Addition of Vhh-TAFI-a51 or Vhh-TAFI-i103 to plasma in the presence of TAFI-TI did not prolong lysis times in an *in vitro* clot lysis assay. The absence of prolongation under those conditions is somehow to be expected as the massive amounts of active TAFIa generated during clot lysis will obscure any possible effect of an increased zymogen activity. Importantly, stimulation of the zymogen activity of the non-activatable TAFI-TI-R92A variant in TAFI depleted plasma resulted in a significant antifibrinolytic effect. This strongly suggests that the zymogen activity, identified as the capability to cleave the small substrate hippuryl-arginine, also represents the ability to remove C-terminal lysines from the fibrin surface. This is further substantiated by the observation that a high level of zymogen activity, obtained by addition of higher concentrations of the non-activatable TAFI-TI-R92A variant also leads to a prolongation of clot lysis. It is of interest to note that it has been suggested that the flexibility of the activation peptide contributes significantly to the zymogen activity [156]. It is therefore tempting to speculate that the increased zymogen activity observed in the TAFI-TI-R92A variant is due to an increased flexibility of the activation peptide consequent to the replacement of an arginine to an alanine at the hinge between the activation peptide and the catalytic moiety. This increased flexibility on its turn also explains the instability of the zymogen activity of this variant.

Screening of diverse panel of TAFI mutants (with mutations present throughout different regions of TAFI) revealed that both Asp^{75} and Thr^{301} are involved in the stimulation of the zymogen activity by the nanobodies. Taking the three-dimensional structure (Fig. 2.3) into consideration, it is very unlikely that the Nbs (with 24 Å × 40 Å dimensions) would bind simultaneously to Asp^{75} and Thr^{301} which are 39 Å apart from each other. Probably Vhh-TAFI-a51 and Vhh-TAFI-i103 bind to either Asp^{75} or Thr^{301}. Since the nanobodies are not able to stimulate the zymogen activity of mouse or rat TAFI, it was possible to deduce the binding region using chimeras. Preliminary experiments (data not shown) evaluating functional effects of the nanobodies on various chimeras (mouse/human and rat/human) indicated that the binding region of the nanobodies is situated between Ile^{67} and Ala^{160}. This suggests that the primary binding site would comprise Asp^{75}. If the actual binding site of the nanobodies is

located in the activation peptide at Asp^{75}, we speculate that binding of the nanobody might result in a translocation of the activation peptide, leading to an increased accessibility of the catalytic site. The concomitant disruption of the stabilizing interactions between residues Val^{35} and Leu^{39}, in the activation peptide, and Tyr^{341} in the dynamic flap region (residues 296-350) [55] might explain the rather short half-life of the stimulated zymogen. Since part of the dynamic flap is involved in the catalytic cleft wall, changes in the dynamic flap could explain the different enzymatic properties of the stimulated zymogen. Impairment of nanobody binding consequent to a mutation at position 301 could possibly be explained by allosteric conformational changes transferred to the activation peptide.

Acknowledgements

Part of this chapter was already described in the Ph.D. thesis of Dr. Karlien Buelens entitled as ″Thrombin activatable fibrinolysis inhibitor: Effect of glycosylation and generation of nanobodies″ (defended on 20th September 2010).

Contributions of authors:

KB generated and characterized a panel of Nbs towards TAFI. NM generated TAFI variants, further explored the features of the Nbs, performed the analyses of selected Nbs on the TAFI (variants), interpreted the data, performed statistical analysis and drafted the manuscript. Epitope mapping and affinity analysis was done by ST and GC. PJD and AG designed research, interpreted the data and reviewed the manuscript.

CHAPTER 3

Identification and Characterization Of Monoclonal Antibodies That Impair the Activation Of Human TAFI Through Different Mechanisms

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Niraj Mishra, Ellen Vercauteren, Jan Develter, Riet Bammens,

Paul J. Declerck, Ann Gils

Laboratory for Therapeutic and Diagnostic Antibodies,

Faculty of Pharmaceutical Sciences,

Katholieke Universiteit Leuven, Leuven

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3.1. Abstract

Background: Thrombin activatable fibrinolysis inhibitor (TAFI) forms a molecular link between coagulation and fibrinolysis and is a putative target to develop profibrinolytic drugs.

Objectives: To evaluate the inhibitory effect of anti-human TAFI monoclonal antibodies (MA) which inhibit the activation of human TAFI through different mechanisms.

Methods and results: Out of a panel of MA raised against TAFI-ACIIYQ, we selected MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 which revealed high affinity towards human TAFI-TI-wt. MA-TCK11A9 was able to inhibit mainly plasmin-mediated TAFI activation, MA-TCK22G2 inhibited plasmin- and thrombin-mediated TAFI activation and MA-TCK27A4 inhibited TAFI activation by plasmin, thrombin and thrombin/thrombomodulin (T/TM) in a dose-dependent manner. These MA did not interfere with TAFIa activity. Using an 8-fold molar excess of MA over TAFI, all three MA were able to reduce clot lysis time significantly i.e. in the presence of exogenous thrombomodulin, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 reduced clot lysis time by 47 ± 9.1%, 80 ± 8.6% and 92 ± 14%, respectively, compared to PTCI. This effect was even more pronounced in the absence of TM i.e. MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 reduced clot lysis time by 90 ± 14%, 140 ± 12% and 147 ± 29%, respectively, compared to PTCI. Mutagenesis analysis revealed that residues at position 268, 272 and 276 are involved in the binding of MA-TCK11A9, residues 147 and 148 in the binding of MA-TCK22G2 and residue 113 in the binding of MA-TCK27A4.

Conclusions: The present study identified three MA, with distinct epitopes, that impair the activation of human TAFI and demonstrated that MA-TCK11A9 which mainly impairs plasmin-mediated TAFI activation can also reduce significantly clot lysis time *in vitro.*

3.2. Introduction

Coagulation and fibrinolytic cascades preserve the integrity of the vascular system and its surrounding tissues. Thrombin activatable fibrinolysis inhibitor (TAFI; EC 3.4.17.20), also known as plasma procarboxypeptidase B (proCPB), procarboxypeptidase R (proCPR) and procarboxypeptidase U (proCPU), provides a very delicate balance between coagulation and fibrinolysis and acts as a molecular link between them [38, 157, 158]. TAFI is a zinc-dependent metallocarboxypeptidase of 401 amino acids. It is produced in the liver as a pre-proenzyme and secreted into circulation as a highly glycosylated zymogen of 56 kDa. Activation of TAFI by enzymes, such as trypsin, plasmin, thrombin or the thrombin–thrombomodulin (T/TM) complex generates a 20 kDa activation peptide (Phe1-Arg92) and a 36 kDa catalytic fragment (TAFIa, Ala93-Val401) [19, 20, 159, 160]. TAFIa is unstable and its activity is downregulated by a temperature-dependent conformational change [37, 51, 161]. This instability differs between human isoforms as well as among species [35, 49].

TAFIa removes carboxy-terminal lysine and arginine-residues from partially degraded fibrin. Consequently, it impedes the fibrin-plasminogen-tissue-type plasminogen activator (t-PA) interaction, resulting in a decreased formation of plasmin and subsequent attenuation of fibrinolysis [38, 63, 162]. Additionally, TAFIa also has substrate specificity towards anaphylatoxins (C3a, C5a), bradykinin, annexin ll and osteopontin, suggesting its probable role in blood pressure, cell migration, inflammation and sepsis [25, 57, 58, 60, 61].

Arterial and venous thromboembolism are one of the major causes of illness and death in the western world. Today, the available therapy is associated with a risk of severe bleeding and needs close lab-monitoring [62]. Therefore, there is a need for the development of clinically safe and easy to use drugs. Considering the fact that TAFI plays a pivotal role in fibrinolysis, while the coagulation cascade remains unaffected [62], TAFI based therapies may result in less bleeding complications. Therefore, development of TAFI inhibitors as profibrinolytic agents seems to be a good strategy to prevent/treat thromboembolism.

Up till now no endogenous TAFIa inhibitor has been reported in plasma [28]. Various unspecific inhibitors [chelating agents such as o-phenantroline and EDTA; reducing agents like dithiothreitol (DTT) and 2-mercaptoethanol] and specific inhibitors [small synthetic substrate analogues such as 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA) and 2-guanidinoethylmercaptosuccinic acid (GEMSA) as well as natural inhibitors like potato tuber carboxypeptidase inhibitor (PTCI), leech carboxypeptidase inhibitor (LCI) and tick carboxypeptidase inhibitor (TCI)] of TAFIa have been reported [28, 54, 62, 63, 85, 87, 159, 163]. Despite of an increasing list of TAFI inhibitors, specificity, poor bioavailability and the biphasic effect observed for some TAFI inhibitors remain a problem [62, 85, 87].

Monoclonal antibodies (MA) are established as candidate drug molecules with high specificity and stability. MA that hamper the activation of TAFI to TAFIa as well as MA that directly inhibit TAFIa activity have been described [30, 88, 89, 164].

This study describes the characterization of three MA that were raised against a stable human TAFI variant (TAFI-ACIIYQ) [53] and that impair the activation of human TAFI through different mechanisms.

3.3. Materials and methods

3.3.1. Materials

All experiments done in this study were performed with the wild-type (wt-) form of human TAFI (TAFI-T^{147}-Ile^{325}, TAFI-TI, without additional tags) unless indicated otherwise. Mouse and rat TAFI wt without additional tags were used throughout the study. Primers for site-directed mutagenesis and sequencing were purchased from Sigma-Aldrich (St Louis, MO, USA). *Pfx*50 DNA polymerase was obtained from Invitrogen (Merelbeke, Belgium). Polymerase chain reactions were carried out with the Mastercycler-Gradient from Eppendorf (Hamburg, Germany). Restriction enzyme *Dpn*I was provided by New England Biolabs (Hertfordshire, UK). Plasmid DNA purification was performed with the Plasmid mini kit I (Omega Bio-Tek, Doraville, GA, USA) and the Nucleobond™ AX500 kit (Machery-Nagel, Düren, Germany). DNA was sequenced by LGC Genomics (Berlin, Germany). Human thrombin, human plasmin, rabbit thrombomodulin and H-D-phenylalanyl-D-prolyl-L-arginine chloromethyl ketone (PPACK) were obtained from Sigma-Aldrich, Enzyme Research Labs (South Bent, UK), American Diagnostica (Greenwich, CT, USA) and Biomol Research Labs (Plymouth Meeting, PA, USA), respectively. Aprotinin, hippuryl-L-arginine and PTCI were obtained from Fluka (Buchs, Switzerland), Bachem (Bubendorf, Switzerland) and Sigma-Aldrich (St Louis, MO, USA), respectively. Tissue-type plasminogen activator (t-PA, Actilyse®) was obtained from Boehringer Ingelheim. Dulbecco′s modified Eagles′ medium, OptiMEM-1 medium containing glutamax and Lipofectamine™ 2000 were purchased from Invitrogen (Merelbeke, Belgium). Horseradish peroxidase (HRP) type VI was purchased from Sigma- Aldrich. Human plasma was collected in-house from 27 healthy volunteers with written consent and pooled for clot lysis. TAFI-depleted plasma was obtained using MA-T4E3 coupled Sepharose 4B as described earlier [155].

3.3.2. Methods

3.3.2.1. Generation of TAFI variants

In the present study, the pcDNA3.1(+) vector (Invitrogen) with the cDNA encoding for TAFI-T^{147}-Ile^{325} (pcDNA-TAFI-TI) was used as the template for mutagenesis [33]. Mouse TAFI and rat TAFI were produced as described before [35, 88] and purified using MA-RT36A3F5-sepharose [89, 165]. The TAFI chimeras hum67mu, hum160rat, hum215rat, and hum350rat were generated, produced and purified as described previously [88, 89].

The binding region of MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 on TAFI was determined using TAFI chimeras. Amino acid sequences of human, mouse and rat TAFI (NM_001872.3, NM_019775 and NM_053617.2, respectively) were aligned in defined regions (Fig. 3.1) and residues specific to human TAFI were identified and visualized in PyMol (PDB ID; 3D66). All the surface exposed human TAFI specific residues were then mutated by site-directed mutagenesis in pcDNA3.1-TAFI-TI into the corresponding mouse residues or alanine. Residues within a 5Å radius of human TAFI specific residues were also selected and mutated into alanine.

Figure 3.1: Alignment of amino acid residues between human, mouse and rat TAFI for 67-160 and 215-350 residues. Human, mouse and rat TAFI sequences were retrieved from GenBank (accession no NM_001872.3, NM_019775 and NM_053617.2, respectively) and aligned with ClustalW for amino acid residues. Residues highlighted with (**∗**) and indicated in yellow represent human specific residues and residues highlighted as grey illustrate those human residues which are either similar to mouse or rat but not to both. Numbers represent the first and last amino acid residues of TAFI. The region between these residues were used in the alignment.

3.3.2.2. Production and purification of TAFI

TAFI variants were transfected in HEK293T cells using Lipofectamine 2000 as per manufacturer's instruction. Culture supernatant was harvested after 96 hours of transfection and stored at -80°C till purification. Human TAFI variants were purified by immunoaffinity chromatography using MA-T27G4 [33] coupled Sepharose beads. Just before use, beads were washed with PBS, mixed with culture supernatant and incubated overnight at 4°C. The packed column was equilibrated and washed with PBS. Then, bound TAFI was eluted with 0.1M glycine (pH 2.7) and fractions were collected on 100µl 1M Tris (pH 9.0). The fractions containing TAFI were pooled and dialyzed against dialysis buffer (20mM Tris, pH 7.4, 100mM NaCl and 10% glycerol). The purity of preparations was determined by 10-15% SDS-PAGE and protein concentration of the purified TAFI was determined spectrophotometrically at 280 nm (ε = 1.28 X10^5 M^{-1} cm^{-1}) [20].

3.3.2.3. Monoclonal antibodies

A panel of 54 MA was raised towards recombinant TAFI- A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335} (TAFI-ACIIYQ) [53] in TAFI deficient mice [60] as described previously [33]. MA were purified from hybridoma supernatants using protein A chromatography [33, 166] and corresponding HRP-conjugated MA were produced as described by Nakane and Kawaoi [167]. Based on preliminary results on their mode of TAFI inhibition and potency, three antibodies MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4, were selected for further characterization.

Cross-reactivity of MA with recombinant TAFI-TI, TAFI-TI variants, rat, mouse and chimeric TAFI was determined with a one-side ELISA using the respective TAFI antigen for capture and rabbit

anti-mouse-HRP for detection as described previously [88]. Data are presented as mean values of percentage reactivity of the MA towards the TAFI variants compared to their reactivity towards TAFI-TI (set at 100%). Values ≤5% are considered as non-reactive. MA-T12D11 was used as a control antibody [88].

3.3.2.4. Affinity Measurements by SPR

Affinity constants (K_A) for the binding of TAFI variants to MA were determined by surface plasmon resonance (SPR) analysis using the Biacore 3000 analytical system (Biacore AB, Uppsala, Sweden) equipped with a CM-5 sensor chip as described [88]. In brief, MA were covalently coupled to approximately 1200 resonance units using a concentration of 5 µg/mL in acetate buffer (10 mM pH 4.5). TAFI was diluted in HBS-EP buffer (Biacore) to concentrations between 5 and 200 nM and injected at a flow rate of 30 µL/min. After each cycle, the chip was regenerated with 10 µL of 10 mM glycine solution (pH 1.5). Analysis of the association and dissociation phases was performed using the Biacore 3000 software (Langmuir binding, local fit). All experiments were performed at least three times independently.

3.3.2.5. Evaluation of the overall inhibitory effect of MA on TAFI activation and TAFIa activity

The overall inhibitory effect of MA on TAFI activation and TAFIa activity was measured as described earlier [90]. Purified TAFI [45 nM (final concentration) in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, + 0.1% BSA pH 7.4)] was incubated (10 min at 37°C) with either buffer or MA (0- to 32-fold molar ratio over TAFI) prior to activation by plasmin (final concentration: 500 nM), in the presence of $CaCl_2$ (final concentration: 5 mM). After 10 min at 22 °C, aprotinin (1.25 µM) and the substrate hippuryl-L-arginine (final concentration: 4 mM) was added to the activation mixture and substrate conversion was allowed to proceed for 30 min at 22 °C. Reactions (100 µL) were stopped by addition of 20 µL of 1N HCl followed by 20 µL of 1N NaOH. Then, 25 µL of 1M Na_2HPO_4 (pH 7.4) and 30 µL of 6% cyanuric chloride (dissolved in 1,4-dioxane) were added and the mixture was vortexed and centrifuged. 100 µL of the supernatant was transferred into a 96-well microtiter plate and the absorbance at 405 nm was measured. Within the concentration range used, a linear correlation between the generated TAFIa activity and colour development was observed. From the comparison of the enzymatic activity of TAFIa generated in the absence and in the presence of MA, the inhibiting capacity of the MA was calculated and expressed as percentage inhibition. Reduced TAFIa activity as detected in this experimental setup can be caused either by interference with the activation mechanism or by direct interference with the TAFIa enzymatic activity.

Evaluation of the effect of the MA on T/TM-mediated TAFI activation was performed as described above, with some modifications. Thrombin (final concentration: 20 nM), and thrombomodulin (final concentration: 5 nM) was used instead of plasmin and aprotinin was replaced by PPACK (final concentration: 37.5 µM) to stop T/TM-mediated activation. Substrate conversion was allowed to proceed for 10 min at 22°C instead of 30 min.

Thrombin is a very weak activator of TAFI and a too prolonged activation time is counteracted by the instability of TAFIa. To counter this problem for evaluation of thrombin-mediated TAFI activation, the stable TAFI variant TAFI-ACIIYQ was used instead of TAFI-TI. A higher amount of thrombin (final concentration: 100 nM) was used in the absence of thrombomodulin and correspondingly a high concentration of PPACK (final concentration: 187.5 mM) was used to stop the reaction. The activation reaction and substrate conversion were allowed to proceed for 120 min at 22°C and 10 min at 22°C, respectively.

3.3.2.6. Evaluation of the direct inhibitory effect of MA on TAFIa activity

The direct inhibitory effect of MA on the TAFIa activity was measured as described previously [90]. Purified TAFI (45 nM in HEPES buffer) was activated by thrombin (final concentration: 20 nM) and thrombomodulin (final concentration: 5 nM) with $CaCl_2$ (final concentration: 5 mM) for 10 min at 22 °C. The activation was stopped by adding PPACK (final concentration: 37.5 µM). Subsequently, either buffer or MA (16-fold molar ratio over TAFI) was added and incubated for 10 min at 37 °C followed by addition of the substrate hippuryl-L-arginine (final concentration: 4 mM). Substrate conversion was allowed to proceed for 10 min 22°C. Stopping the reaction, colour development and calculation of percentage inhibition was carried out as described above. Reduced TAFIa activity as detected in this experimental setup is the consequence of a direct interference of the MA with the enzymatic activity of TAFIa.

3.3.2.7. Evaluation of the effect of the MA on the activation of TAFI

The effect of the MA on the activation of TAFI to TAFIa was evaluated as described earlier [90]. Briefly, TAFI (concentration at activation step: 0.41 µM in Tris buffer; 20 mM Tris, 100 nM NaCl, pH 7.4) was incubated with either buffer or MA (3-fold molar ratio over TAFI) for 10 min at 37°C prior to activation with either thrombin (23 nM)/thrombomodulin (5.8 nM) or plasmin (417 nM) in the presence of $CaCl_2$ (5.8 mM). The reactions were stopped by adding PPACK (34.5 µM) or aprotinin (1.2 µM), respectively, and SDS (1% final concentration) to the reaction mixtures followed by heating for 30 sec at 100°C. The generated fragments were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using PhastGelTM gradient 10-15% gels, followed by silver staining.

3.3.2.8. Evaluation of the effect of MA during *in vitro* clot lysis

The clot lysis assay was performed in a 96-well microtiter plate as described previously [90]. Pooled plasma (final concentration: 30%) was mixed either with dilution buffer (10 mM Tris, 0.01% tween 20, pH 7.5) or with MA (final concentration: 432 nM, resulting in an 8-fold molar ratio over TAFI assuming a concentration of 180 nM TAFI in plasma), or PTCI (20 µg/ml, final concentration). The plate was incubated at 37°C for 10 min and exogenous thrombomodulin (1 nM) and t-PA (120 pM) were added. Then clot formation was induced by addition of $CaCl_2$ (10.6 mM) and the plate was read at 2 min intervals at 405 nm at 37°C. The 50% clot lysis time was determined, defined as the time interval between the time point of full clot formation to the midpoint of the maximum turbidity to clear transition. Reduction of the clot lysis time was calculated relative to the reduction of clot lysis time in the absence (0% reduction) and presence of PTCI (100% reduction). Alternatively, this assay was carried out

without the addition of exogenous thrombomodulin. Clot-lysis assays were also performed in TAFI depleted plasma in presence and absence of TM.

3.3.2.9. Measurement of TAFIa activity during clot formation and lysis

The clot lysis assay was performed in a 96-well microtiter plate as described above with some modifications [90, 155]. Pooled plasma (final concentration: 30%) was mixed either with buffer (20mM HEPES, 0.01% tween 20; pH; 7.4) or with MA (final concentration: 432 nM). t-PA (final concentration: 353 pM) was added and clot formation was induced by addition of $CaCl_2$ (10.6 mM). 100µl of this mix was placed in the microtiter plate and the plate was read at 2 min intervals at 405 nm at 37°C. In parallel/Simultaneously for analysis of TAFIa activity at different timepoints 200µl of this mix for every timepoint was incubated in separate tubes at 37°C and after every 10 min, one tube was taken out and PPACK (final concentration: 41 µM) and aprotinin (final concentration: 1.4 µM) was added to inhibit all thrombin and plasmin activity, respectively. Samples were stored further on ice until the carboxypeptidase activity was assayed.

TAFIa activity was measured using the chromogenic assay as described above with some modifications. In order to distinguish between TAFIa and CPN activity, the samples were analyzed in the presence and absence of PTCI (final concentration: 28 µg/ml). 80µl of each sample was mixed either with HEPES buffer [25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, + 0.1% BSA pH; 7.4] or PTCI and incubated for 10 min at 22°C. Then the substrate hippuryl-L-arginine (final concentration: 5 mM) was added to the mix and substrate conversion was allowed to proceed for 10 min at 22°C. Stopping the reaction, colour development and measurement of optical density was carried out as described above. Values obtained in the presence of PTCI were subtracted from total values (without PTCI) to calculate the specific TAFIa activity in plasma and TAFIa activity was calculated in U/L [OD (TAFIa)/ m (slope) X 10 (time-min) = U/L]. Hippuric acid was used as standard to calculate TAFIa activity. To evaluate the effect of MA-TCK11A9 in the presence of plasmin inhibitor; aprotinin (final concentration: 1µM) was added at the start of the experiment.

3.3.2.10. Statistical analysis

Quantitative data were represented as mean and standard deviation (SD). The data were analyzed using paired two-tailed t-test with Graph-Pad Prism (GraphPad, Inc. San Diego, CA, USA). The two-tailed p-value was calculated and $p < 0.05$ was considered statistically significant. Dose–response curves were fitted to one-side binding hyperbolas.

3.4. Results

3.4.1. Reactivity of MA towards human, mouse and rat TAFI

MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 were generated towards the stable human TAFI variant TAFI-ACIIYQ. One-side ELISA results revealed that these three antibodies cross-react with human wild-type TAFI but not with mouse or rat TAFI (Table 3.1A). The reactivity of MA-TCK22G2 and MA-TCK27A4 towards TAFI-TI and TAFI-ACIIYQ was confirmed by surface plasmon resonance (SPR) analysis, in which MA-TCK22G2 revealed a high affinity towards TAFI-ACIIYQ ($K_A = 22 \pm 5.5 \times 10^8\ M^{-1}$) and TAFI-TI ($K_A = 1.5 \pm 0.5 \times 10^8\ M^{-1}$). MA-TCK27A4 showed high affinity towards TAFI-ACIIYQ ($K_A = 16 \pm 4.7 \times 10^8\ M^{-1}$) and TAFI-TI ($K_A = 12 \pm 4.7$ X $10^8\ M^{-1}$). MA-TCK22G2 and MA-TCK27A4 revealed no affinity ($KA < 10^6\ M^{-1}$) towards mouse and rat TAFI (Table 3.2A). The affinity of TAFI variants towards MA-TCK11A9 could not be determined via SPR analysis due to unknown reasons.

Table 3.1: Percentage reactivity of MA towards human, mouse and rat TAFI variants compared to the reactivity of MA towards TAFI-TI.

	TAFI-variants	MA-T12D11	MA-TCK11A9	MA-TCK22G2	MA-TCK27A4
A	TAFI-ACIIYQ	71	180	153	140
	TAFI-TI	100	100	100	100
	Mouse TAFI	<5	<5	<5	<5
	Rat TAFI	<5	<5	<5	<5
B	TAFI-TI-F113A	86	45	56	<5
	TAFI-T147R-I	97	51	<5	52
	TAFI-TI-A148I	86	91	<5	75
	TAFI-TI-K268A	97	13	81	72
	TAFI-TI-S272D	91	<5	86	80
	TAFI-TI-R276A	81	<5	53	53
	TAFI-TI-K268A-S272D	97	<5	74	65
	TAFI-TI-S272D-R276A	87	<5	104	86
	TAFI-TI-K268A-S272D-R276A	98	<5	84	67

MA-T12D11; control antibody.

Table 3.2: Affinity constants (K_A; $10^8\ M^{-1}$) of human, mouse and rat TAFI and human TAFI variants towards MA-TCK22G2 and MA-TCK27A4.

	TAFI-variant	MA-TCK22G2	MA-TCK27A4
		K_A (M^{-1})	K_A (M^{-1})
A	TAFI-ACIIYQ	22±5.5	16±4.7
	TAFI-TI	1.5±0.5	12±4.7
	Mouse	NB	NB
	Rat	NB	NB
B	TI-F113A	1.2±0.5	NB
	T147R-I	NB	8.6±2.3
	TI-A148I	NB	10±3.4
	TI-K268A	1.6±0.4	11±3.0
	TI-S272D	1.5±0.4	9.9±2.9
	TI-R276A	0.9±0.3	5.8±1.3
	TI-K268A-S272D	1.3±0.5	7.9±3.1
	TI-K268A-R276A	1.3±0.5	8.5±2.1
	TI-K268A-S272D-R276A	1.4±0.7	9.0±3.1

Mean ± SD, n≥3. NB (no binding), $KA \leq 10^6$.

3.4.2. Direct inhibition of TAFIa activity

Evaluation of the effect of MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 on the activity of TAFIa revealed that neither of the MA affected TAFIa activity (Table 3.3).

Table 3.3: Effect of MA on TAFIa activity, TAFI activation and on 50% clot lysis time

	% reduction of TAFI activation††			% reduction of TAFIa activity†	% reduction of clot-lysis time††	
MA	**T/TM**	**Plasmin**	**T**		**Expressed relative to % reduction by PTCI (+TM)**	**Expressed relative to % reduction by PTCI (-TM)**
TCK11A9	<2	64 ± 4.3	<2	<2	47 ± 9.1*	90 ± 14*
TCK22G2	<2	58 ± 4.0	51 ± 1.5	<2	80 ± 8.6*	140 ± 12*
TCK27A4	98 ± 2.0	100 ± 0.4	96 ± 2.1	<2	92 ± 14*	147 ± 29*

Mean ± SD, n ≥ 3; * p < 0.05 vs. no TAFI inhibitor. † evaluated at 16-fold molar ratio of MA over TAFI. †† evaluated at 8-fold molar ratio of MA over TAFI

3.4.3. Impairment of the activation of TAFI to TAFIa

To determine a possible effect of the MA on the activation of TAFI, MA were added prior to the activation of TAFI by plasmin, thrombin or the T/TM complex. MA-TCK11A9 inhibits plasmin-mediated TAFI activation in a dose dependent manner i.e. 64 ± 4.3% at an 8-fold molar ratio of MA over TAFI, with a maximum inhibition of 84 ± 2.5% and a corresponding IC_{50} value of 118 nM but does not affect the thrombin or T/TM-mediated activation of TAFI (Table 3.3, Fig. 3.2A).

Using an 8-fold molar ratio of MA over TAFI, MA-TCK22G2 inhibits both the plasmin- and thrombin-mediated TAFI activation by 58 ± 4.0% and 51 ± 1.5%, respectively, but does not inhibit the T/TM activation of TAFI (Table 3.3, Fig. 3.2B). MA-TCK22G2 revealed a maximum inhibition of plasmin- mediated TAFI activation of 78 ± 2.1% with a corresponding IC50 value of 141 nM and a maximum inhibition of thrombin-mediated TAFI activation of 51 ± 3.3% with a corresponding IC_{50} value of 44 nM.

MA-TCK27A4 inhibits plasmin-, thrombin- and T/TM-mediated TAFI activation by 100 ± 0.4%, 96 ± 2.1% and 98 ± 2.0%, respectively, at an 8-fold molar ratio of MA over TAFI. The maximum inhibition by MA-TCK27A4 was 100 ± 2.6%, 100 ± 3.4% and 100 ± 2.9%, respectively, and the corresponding IC_{50} values were 7 nM, 23 nM and 11 nM, respectively (Table 3.3, Fig. 3.2C).

To confirm the effect of the MA on TAFI activation, fragmentation products of TAFI upon activation of TAFI by plasmin or the T/TM complex in the absence and presence of MA were analyzed using SDS-PAGE. These data confirm that MA-TCK11A9 and MA-TCK22G2 are able to inhibit plasmin-mediated TAFI activation and MA-TCK27A4 inhibits plasmin-, and T/TM-mediated TAFI activation (data not shown).

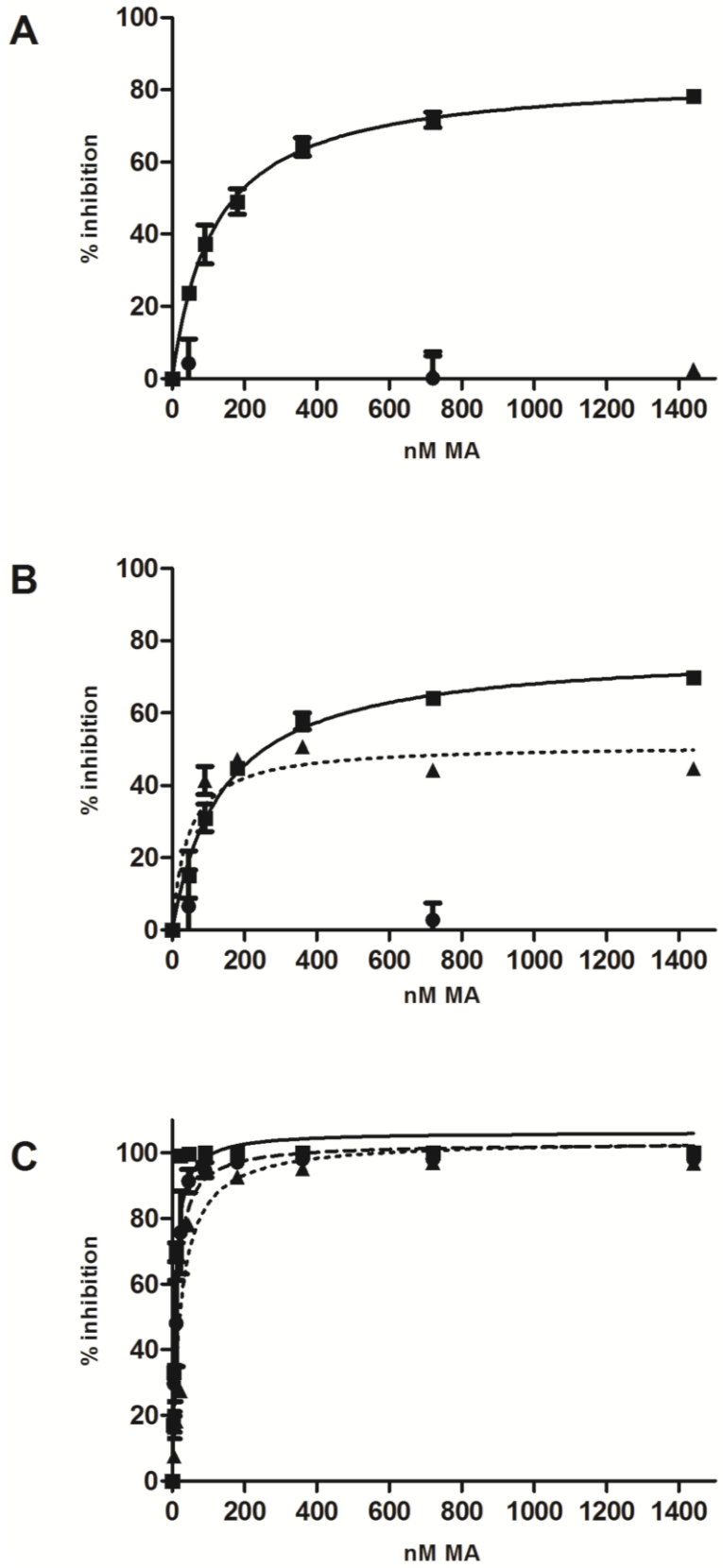

Figure 3.2: Dose-dependent inhibition by MA-TCK11A9 (A), MA-TCK22G2 (B) and MA-TCK27A4 (C) on plasmin- (■), thrombin- (···▲···) and T/TM- (-- ● --)-mediated TAFI activation. Percentage inhibition of TAFI activation was calculated relative to activated TAFI in the absence of MA. The results represent mean ± SD (n ≥ 3).

3.4.4. Effect of MA on clot lysis

To investigate the functional effect of the MA on clot lysis, plasma was preincubated either with buffer (negative control), PTCI (positive control) or MA. In the absence of both MA and PTCI, the clot lysis time was 133 ± 11 min in the presence of 1 nM exogenous thrombomodulin. In the presence of PTCI clot lysis time was shortened to 76 ± 4 min. At an 8-fold molar ratio of MA (i.e. 432 nM) over TAFI, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 significantly accelerated clot lysis to 106 ± 12 min, 88 ± 9 min and 81 ± 5 min, respectively. Compared to PTCI, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 reduced clot lysis times by 47 ± 9.1%, 80 ± 8.6 % and 92 ± 14%, respectively (Table 3.3). The effect in clot lysis was more pronounced in the absence of exogenous thrombomodulin. Under these conditions, in the absence of both MA and PTCI, the clot lysis time was 104 ± 15 min. In the presence of PTCI the clot lysis time was shortened to 68 ± 15 min. At an 8-fold molar ratio of MA (i.e. 432 nM) over TAFI, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 significantly accelerated clot lysis to 71 ± 20 min, 54 ± 13 min and 51 ± 7 min, respectively. Compared to PTCI, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 reduced clot lysis times by 90 ± 14%, 140 ± 12 % and 147 ± 29%, respectively (Table 3.3). None of the antibodies was able to reduce 50% clot lysis time in TAFI depleted plasma (data not shown).

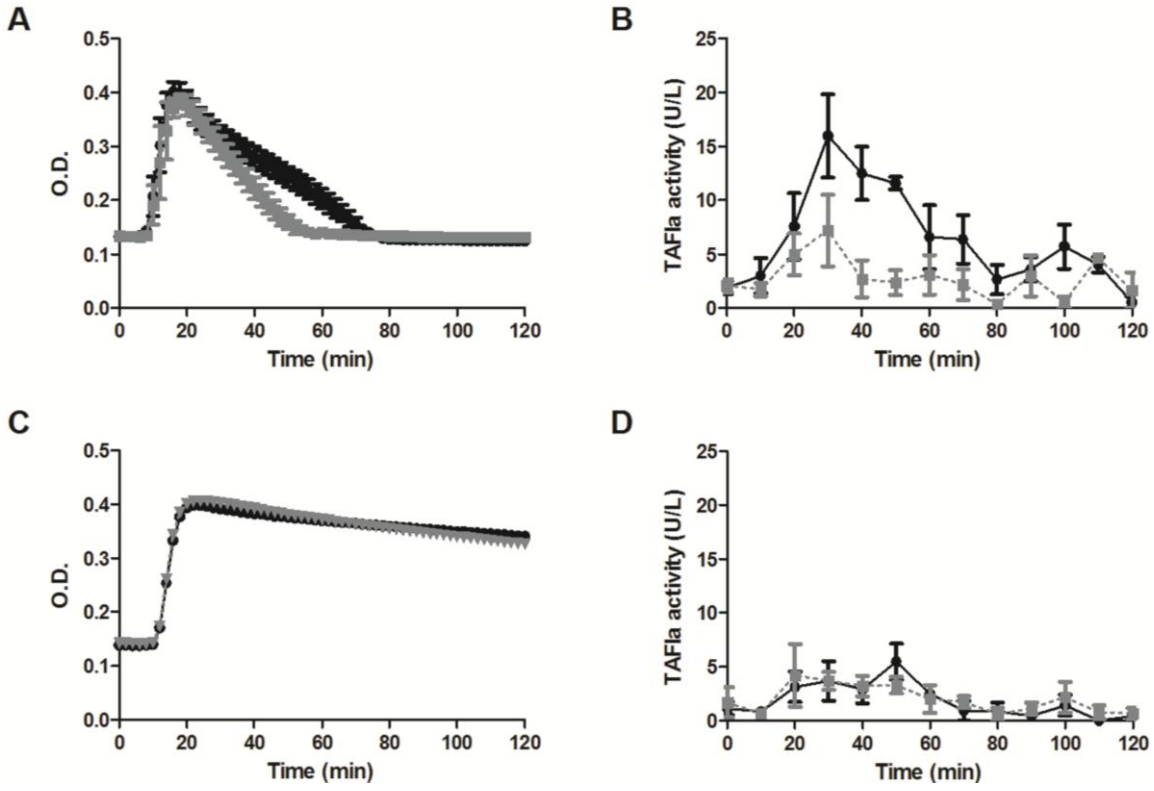

Figure 3.3: TAFIa activity during clot formation and lysis. (A) Clot lysis profile and (B) TAFIa activity (expressed in U/L) during clot lysis in the absence (black curve;– ● –) and in the presence (grey curve;– ■ –) of MA-TCK11A9 and (C) Clot lysis profile and (D) TAFIa activity (expressed in U/L) during clot lysis after addition of 1 µM aprotinin in the absence (black curve;– ● –) and the presence (grey curve;– ■ –) of MA-TCK11A9. The results represent mean ± SD (n = 3).

3.4.5. Effect of MA-TCK11A9 on TAFIa generation during clot formation and lysis

To examine the effect of MA-TCK11A9 on TAFIa activity during clot lysis experiments, we measured TAFIa activity at various timepoints during clot lysis in the presence and absence of MA-TCK11A9. Under these experimental conditions, only a small second TAFIa activity peak could be observed in the absence of MA-TCK11A9 (Fig. 3.3B). Upon addition of MA-TCK11A9, we observed a significant reduction in clot lysis time (Fig. 3.3A) as well as a reduction in both TAFIa activity peaks (Fig. 3.3B). To further investigate the effect of MA-TCK11A9 on TAFIa generation in the absence of plasmin, we added 1 µM aprotinin (final concentration) in the beginning of the clot lysis experiments. Consequently, no clot lysis occurred (Fig. 3.3C) and a strongly decreased amount of both TAFIa activity peaks was observed (Fig. 3.3D). Addition of MA-TCK11A9 did not affect the clot lysis time nor the amount of generated TAFIa activity (Fig. 3.3C and 3.3D).

3.4.6. Inhibitory effect of MA towards human/mouse and human/rat TAFI chimeras

The inhibitory effect of the MA on the plasmin-mediated activation of human, mouse, rat, human/mouse and human/rat TAFI chimeras was investigated using a 16-fold molar ratio of MA over TAFI (Table 3.4). MA-TCK11A9 was able to inhibit plasmin-mediated activation of the hum350rat TAFI chimera but not of other chimeras whereas both MA-TCK22G2 and MA-TCK27A4 were able to inhibit plasmin-mediated activation of hum160rat, hum215rat and hum350rat TAFI chimeras but not the hum67mu chimera. These results were confirmed by one-side ELISA (data not shown).

From these data, it can be concluded that the epitope of MA-TCK11A9 resides within the 215-350 amino acid region whereas the epitope of MA-TCK22G2 and MA-TCK27A4 resides within the 67-160 amino acid region.

Table 3.4: Percentage inhibition of the MA on the plasmin-mediated activation of human, mouse and rat TAFI variants and TAFI chimeras

TAFI variants	MA-TCK11A9†	MA-TCK22G2†	MA-TCK27A4†
TAFI-TI	72 ± 4	64 ± 1	100 ± 0
TAFI-ACIIYQ	91 ± 2	81 ± 1	100 ± 0
Mouse TAFI	15 ± 1	17 ± 6	12 ± 7
Rat TAFI	<5	9 ± 5	17 ± 10
hum67mu	16 ± 9	9 ± 8	12 ± 7
hum160rat	<5	64 ± 4	99 ± 1
hum215rat	6 ± 2	71 ± 3	100 ± 1
hum350rat	72 ± 2	70 ± 6	99 ± 1

Mean ± SD, n ≥ 3. † Molar ratio, MA:TAFI = 16

3.4.7. Affinity and inhibitory effect of MA towards human TAFI variants

The 67-160 amino acid and the 215-350 amino acid regions of mouse, rat and human TAFI were aligned (Fig. 3.1). TAFI was visualized in PyMol (PDB ID; 3D66) and surface exposed human TAFI specific residues were replaced either by the corresponding mouse residue or by alanine. To elucidate the epitope of MA-TCK11A9, the following mutations were introduced i.e. F222A, Y223H, A224K, H227R-I229V, E245K, S272D, Q280H, H293Q-V295L, T301N, K324S-S326N and H335S-T339S in

TAFI-TI. To elucidate the epitope of MA-TCK22G2 and MA-TCK27A4, the following mutations were introduced i.e. C69F-S70N, L73M, A74N, D75N, I84T, F113V, R117Q, T123Q, H126Y, T147R and A148I in TAFI-TI. All mutants were generated, expressed and conditioned medium was tested for TAFIa activity upon activation by plasmin (data not shown). All mutants were fully activatable by plasmin, except TAFI-TI-H126Y.

The effect of MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 on the plasmin-mediated activation of the generated mutants was assayed on conditioned medium. Based on the reduction in percentage inhibition of the MA on the plasmin-mediated activation, the following TAFI variants were selected for purification and characterization i.e. TAFI-TI-S272D for MA-TCK11A9, TAFI-T147R-I and TAFI-TI-A148I for MA-TCK22G2, TAFI-TI-F113A for MA-TCK27A4.

MA-TCK11A9 inhibited plasmin-mediated activation of TAFI-TI by 64 ± 4.3% (at 360 nM) and of TAFI-TI-S272D by 22 ± 3.3% (at 360 nM). These results revealed that the plasmin-mediated activation of TAFI-TI-S272D is significantly less inhibited by MA-TCK11A9. To identify other residues involved in the epitope of MA-TCK11A9, five surface exposed residues within the radius of 5Å of S^{272} were selected and replaced by alanine (except for Ala^{269}) generating the following TAFI variants i.e. TAFI-TI-K268A, TAFI-TI-A269S, TAFI-TI-F273A, TAFI-TI-R275A and TAFI-TI-R276A. Conditioned medium was tested for TAFIa activity. All mutants except TAFI-TI-F273A were fully activatable by plasmin (data not shown). Based on the reduction in percentage inhibition of the MA on the plasmin-mediated activation, TAFI-TI-K268A and TAFI-TI-R276A were selected for purification and characterization. A dose-dependent effect of MA-TCK11A9 on plasmin-mediated activation of TAFI-TI-K268A and TAFI-TI-R276A was evaluated (Fig. 3.4A). MA-TCK11A9 inhibited plasmin-mediated activation of TAFI-TI-K268A by 12 ± 6.9% (360 nM) and TAFI-TI-R276A by <2% (360 nM) (Fig. 3.4A). Subsequently, two double mutants i.e. TAFI-TI-K268A-S272D and TAFI-TI-S272D-R276A and one triple mutant i.e. TAFI-TI-K268A-S272D-R276A, were generated, purified and subjected to the inhibitory assay revealing 9 ± 4.9%, 18 ± 7% and 18 ± 11% inhibition of plasmin-mediated TAFI activation by MA-TCK11A9 (360nM) (Fig. 3.4A). Binding of TAFI or TAFI mutants to MA-TCK11A9 could not be determined by SPR due to unknown reasons. One-side ELISA analysis (Table 3.1B) revealed a 87% lower reactivity of TAFI-TI-K268A and a 100% lower reactivity of TAFI-TI-R276A, TAFI-TI-S272D, TAFI-TI-K268A-S272D, TAFI-TI-S272D-R276A and TAFI-TI-K268A-S272D-R276A towards MA-TCK11A9. These results identified Lys^{268}, Ser^{272}, and Arg^{276} as major residues interacting with MA-TCK11A9.

The dose-dependent effect of MA-TCK22G2 on the plasmin-mediated activation of TAFI-TI, TAFI-T147R-I and TAFI-TI-A148I was evaluated (Fig. 3.4B). At 360 nM, MA-TCK22G2 inhibited plasmin-mediated activation of TAFI-TI by 58 ± 4.0%, TAFI-T147R-I by 7 ± 7.3% and TAFI-TI-A148I by 4 ± 13% (Fig. 3.4B). In SPR analysis, TAFI-TI revealed a high affinity towards MA-TCK22G2 (K_A = 1.5 ± 0.5 × 10^8), but TAFI-T147R-I and TAFI-TI-A148I failed to show any binding towards this MA (Table 3.2B). Lack of binding of the TAFI-T147R-I and TAFI-TI-A148I to MA-TCK22G2 was confirmed by one-side ELISA experiments (Table 3.1B). These results revealed that MA-TCK22G2 binds to Thr^{147} and Ala^{148}.

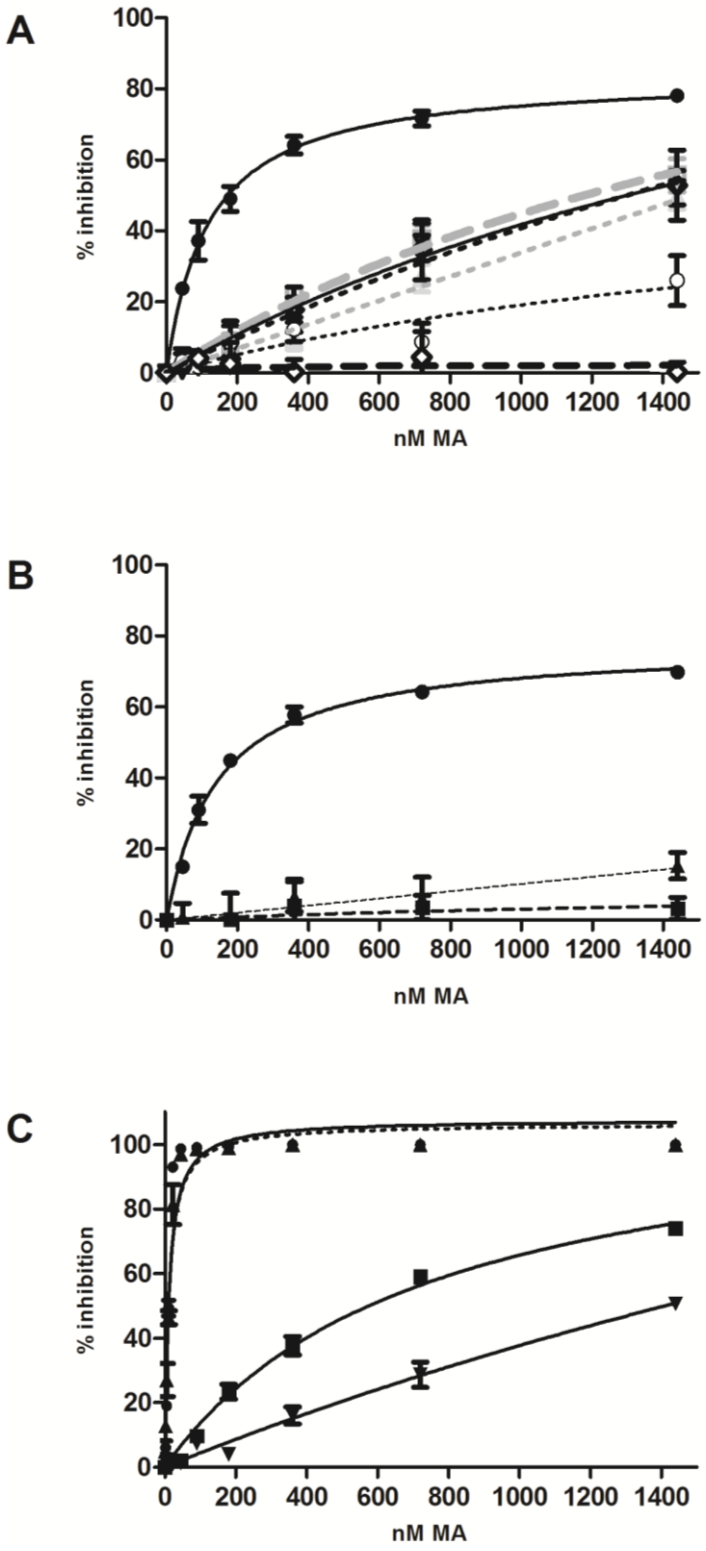

Figure 3.4: Dose-dependent inhibition of activation of human TAFI and its variants by MA. (**A**) Effect of MA-TCK11A9 on plasmin-mediated activation of TAFI-TI (– ● –),TAFI-TI-K268A (···O···),TAFI-TI-S272D (- - ■ - -), TAFI-TI-R276A (**--◇--**), TAFI-TI-K268A-S272D (-- ▲ --),TAFI-TI-S272D-R276A (—▼—) and TAFI-TI-K268A-S272D-R276A (**--♦--**), (**B**) Effect of MA-TCK22G2 on plasmin-mediated activation of TAFI-TI (– ● –),TAFI-T147R-I (··▲··) and TAFI-TI-A148I (-- ■ --) and (**C**) Effect of MA-TCK27A4 on plasmin-mediated TAFI-TI (– ● –) and TAFI-TI-F113A (– ■ –) as well as T/TM-mediated activation of TAFI-TI (--▲--) and TAFI-TI-F113A (– ▼ –). Percentage activation of inhibition of TAFI activation was calculated relative to activated TAFI in the absence of MA. The results represent mean ± SD (n ≥ 3).

The dose-dependent effect of MA-TCK27A4 on plasmin-mediated and T/TM-mediated activation of TAFI-TI and TAFI-TI-F113A was evaluated (Fig. 3.4C). At 360 nM, MA-TCK27A4 inhibited plasmin-mediated activation of TAFI-TI by 100 ± 0.4% and of TAFI-TI-F113A by 38 ± 5.0% and T/TM-mediated activation of TAFI-TI by 98 ± 2.0% and of TAFI-TI-F113A by 16 ± 4.6% (Fig. 3.4C). SPR analysis revealed no binding of TAFI-TI-F113A to MA-TCK27A4 (Table 3.2B) and these results were confirmed by one-side ELISA (Table 3.1B). This revealed Phe^{113} as the major residue in the epitope of MA-TCK27A4.

3.5. Discussion

TAFIa exerts an antifibrinolytic effect by removing C-terminal lysines from partially degraded fibrin degradation products, thereby abolishing their cofactor function in the activation of plasminogen by t-PA [38, 63, 162]. Many clinical studies have correlated TAFI antigen levels with cardiovascular events such as angina pectoris [168], coronary artery disease [169], ischemic stroke [170], myocardial infarction [73] and venous thrombosis [69]. Considering TAFI as a molecular link between thrombosis and coagulation, the drawbacks of presently available antithrombotic agents/therapy and the large population suffering from thrombosis disorders, the development of highly specific TAFI inhibitors is a hot topic [62, 171, 172]. The use of TAFI inhibitors as an adjuvant in thrombolytic therapy would allow lower amounts of infused plasminogen activators and consequently enhance thrombolytic efficiency and reduce side effects. Alternatively, TAFI inhibitors could also be used in the prevention of thrombotic events. Although a lot of basic research on TAFI has been performed, the complete *in vivo* mechanism of TAFI activation by various activators and their relative contributions in various pathways is lacking. MA that inhibit TAFI activation by various mechanisms (thrombin, the T/TM complex and/or plasmin) are excellent tools to study the mechanism of TAFI activation, regulation and its role in physiological conditions.

In the present study, we characterized three MA that inhibit the activation of TAFI. MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 were raised towards a stable variant of human TAFI. Although raised towards the stable TAFI variant (TAFI-ACIIYQ) containing five mutations, all three MA revealed a high affinity towards human TAFI-TI-wt. These three MA revealed no affinity towards rat or mouse TAFI (Table 3.1A and Table 3.2). None of these MA interfered with TAFIa activity (Table 3.3). MA-TCK11A9 is able to inhibit plasmin-mediated activation, MA-TCK22G2 inhibits plasmin- and thrombin-mediated TAFI activation and MA-TCK27A4 inhibits TAFI activation by plasmin, thrombin and T/TM (Table 3.3 and Fig. 3.2). MA that inhibit TAFI through the impairment of TAFI activation were previously reported by Gils *et. al.*, Bajzar *et. al*. and Bouma *et. al* [30, 88, 164]. However, these previously reported MA impair mainly T/TM- as well as plasmin-mediated TAFI activation. MA that impairs mainly the plasmin-mediated TAFI activation have not been reported yet. However, nanobodies with a comparable mechanism of action were reported recently [90].

An association between TAFI antigen levels and *in vitro* clot lysis time among healthy individuals has been reported [31]. Previous studies also revealed a TAFI-related retardation of t-PA and u-PA mediated clot lysis and reported that this delay is dependent on the type and concentration of the plasminogen activator used [173, 174]. The *in vitro* clot lysis assays performed in this study revealed that all three MA have strong profibrinolytic properties in human plasma. Compared to PTCI, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 reduced clot lysis times by 47 $\pm$ 9.1%, 80 $\pm$ 8.6% and 92 $\pm$ 14% (Table 3.3), when clot lysis experiments were performed upon addition of 1 nM exogenous TM. In the absence of exogenous TM reduction in clot lysis was more pronounced. Compared to PTCI, MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 reduced clot lysis times by 90 $\pm$ 14%, 140 $\pm$ 12% and 147 $\pm$ 29%. Thus all three MA are able to accelerate clot lysis significantly in the presence as

well as in the absence of exogenous thrombomodulin. Surprisingly, MA-TCK11A9 which mainly inhibits plasmin-mediated TAFI activation, is capable of reducing clot lysis time.

A biphasic pattern of TAFI activation during *in vitro* clot lysis was previously shown demonstrating that a first peak of TAFIa activity is generated via T/TM activation whereas a second peak of TAFIa activity is generated via plasmin-mediated TAFI activation [42, 155]. A recent report suggested that even in the absence of thrombomodulin, thrombin but not plasmin, mediates TAFI activation in *in vitro* clot lysis assays [43]. However in these experiments 50% plasma and 40 ng/ml of t-PA was used and aprotinin, a selective plasmin inhibitor, was added after the formation of the first TAFIa activity peak. This resulted in a complete reduction of the second TAFIa activity peak indicating indeed that this second TAFIa activity burst is due to TAFI activation by plasmin. However, we would like to stress that in our clot lysis experiments 30% plasma and 8 ng/ml of t-PA was used. To be able to measure TAFIa activity during clot dissolution we repeated some experiments using 30% plasma with 24 ng/ml t-PA. Under these conditions, only a small second TAFIa activity peak was observed and addition of MA-TCK11A9 resulted into reduction of both TAFIa activity peaks (Fig. 3.3B). This indicates that in these conditions, plasmin activation of TAFI occurs during both clot formation and clot dissolution. This also clarifies our observation i.e. strong reduction of clot lysis time when an inhibitor of plasmin-mediated TAFI activation i.e. MA-TCK11A9 is added (Fig. 3.3A). Under similar conditions in the presence of aprotinin, no clot lysis occurred (Fig. 3.3C), a strong reduction in both TAFIa activity peaks was observed (Fig. 3.3D) and addition of MA-TCK11A9 had no effect on clot lysis (Fig. 3C) nor on the amount of generated TAFIa activity (Fig. 3.3D), confirming that the profibrinolytic effect of MA-TCK11A9 is due to its ability to inhibit plasmin-mediated TAFI activation. All these results strongly demonstrate that T/TM as well as plasmin-mediated TAFI activation contributes to the TAFI activation during clot formation and lysis. This was also recently shown *in vivo* using a mice thromboembolism model in which a monoclonal antibody that mainly inhibiting plasmin-mediated TAFI activation was able to significantly reduce fibrin deposition in lungs upon injection of thromboplastin [81].

Epitope mapping revealed that Lys^{268}, Ser^{272} and Arg^{276} are the major residues responsible for the binding of human TAFI to MA-TCK11A9, Thr^{147} and Ala^{148} are the major residues for the binding of human TAFI to MA-TCK22G2 and Phe^{113} was identified as the major residue responsible for the binding of human TAFI to MA-TCK27A4.

In a previous study, it was shown that MA-T1C10 and MA-T94H3 attenuate the T/TM as well as the plasmin-mediated TAFI activation whereas MA-T12D11 inhibits exclusively T/TM-mediated TAFI activation. The major residues for the binding of MA-T94H3, MA-T12D11 and MA-T1C10 to TAFI were Val^{41}, Gly^{66} and Gln^{45}, respectively [88]. These three residues are located in the activation peptide region (indicated in orange, brown and yellow, respectively in Fig. 3.5). It was recently shown that positively charged amino acid residues (i.e. Lys^{42}, Lys^{43}, Lys^{44}) located in the activation peptide are responsible for binding with TM [175]. The residues identified as major residues in the epitopes of the MA described in the current study are located outside the activation peptide region. Thr^{147} and Ala^{148} (epitope of MA-TCK22G2) are located in the loop that connects the β_2 with the β_3 –strand

whereas Lys^{268}, Ser^{272} and Arg^{276} (epitope of MA-TCK11A9) are located in α_4-helix. Binding to either Thr^{147} and Ala^{148} or Lys^{268}, Ser^{272} and Arg^{276} might induce a conformational change or allosteric modulation in the TAFI molecule in such a way that plasmin and/or thrombin will no longer be able to activate TAFI. Phe^{113} (epitope of MA-TCK27A4) is located in α_1-helix near (8Å) the Arg^{92} cleavage site. Through binding of MA-TCK27A4 to Phe^{113}, Arg^{92} will no longer be accessible to plasmin, thrombin and T/TM.

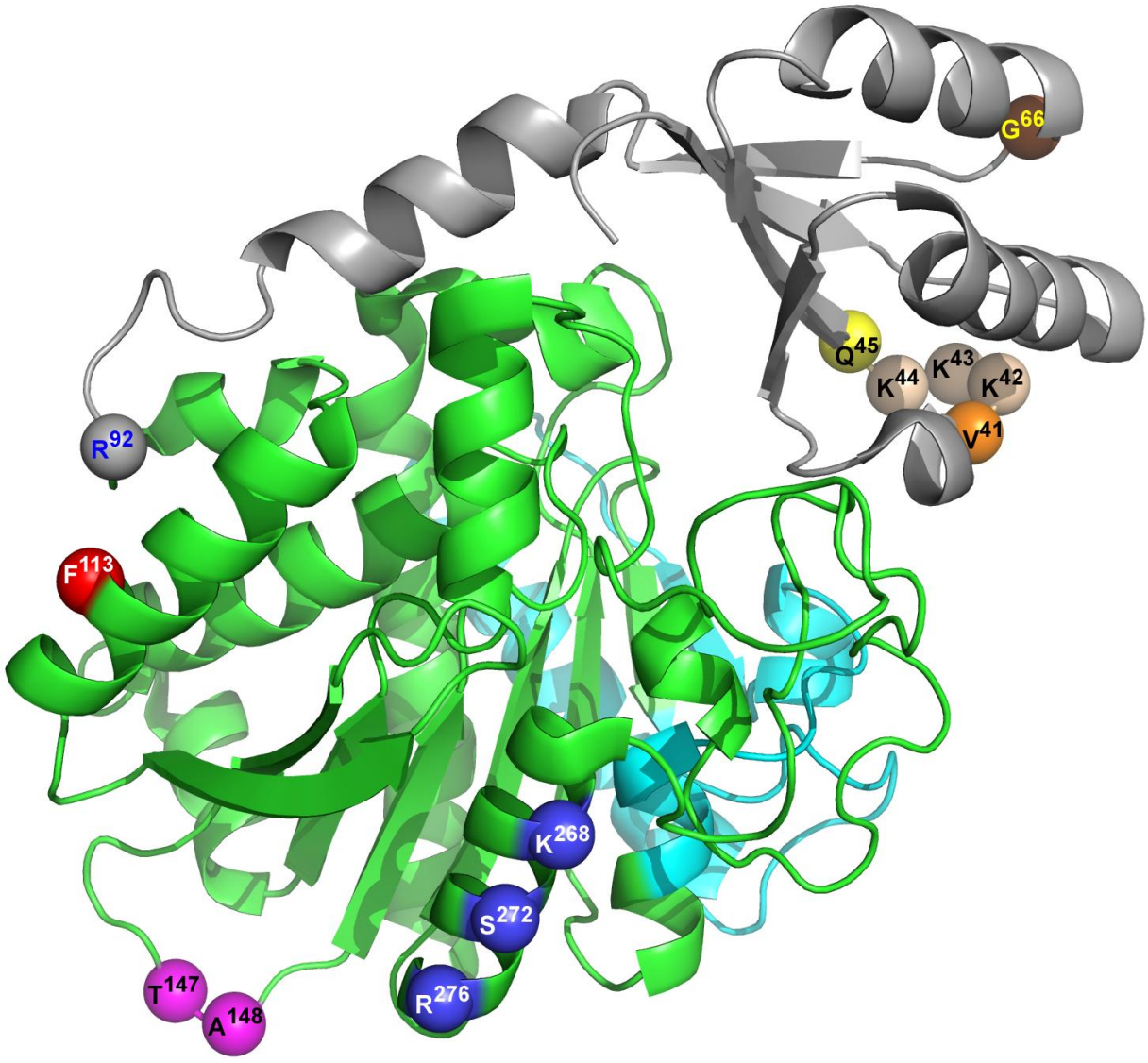

Figure 3.5: Illustration of epitope residues of MA-TCK11A9, MA-TCK22G2 and MA-TCK27A4 on TAFI. TAFI structure was visualized in Pymol using PDB ID: 3D66 [55]. Grey, green and cyan represents the activation peptide, the catalytic domain and the dynamic flap of TAFI, respectively. R^{92} is the cleavage site for plasmin, thrombin and T/TM. The epitope of MA-TCK11A9 (K^{268}, S^{272} and R^{276}), MA-TCK22G2 (T^{147} and A^{148}) and MA-TCK27A4 (F^{113}) are represented in blue, magenta and red colors respectively.

In conclusion, this study identified three MA that impair the activation of TAFI through different mechanisms involving different epitopes. Data obtained with MA-TCK11A9 which mainly impairs plasmin-mediated TAFI activation, reveal that plasmin-mediated activation of TAFI also plays an important role in clot lysis and consequently in fibrinolysis. In addition, the availability of a selective

inhibitor of plasmin-mediated activation of TAFI might have opportunities to be used in other processes like blood pressure (bradykinin), cell migration (annexin ll), infection and inflammation (C3a, C5a and osteopontin) [25, 57, 58, 60, 61] in which activation of TAFI by plasmin might play a role.

Acknowledgement

Part of this chapter was already described in the Ph.D. thesis of Dr. Jan Develter entitled as ″ Bridging anti-TAFI and anti-PAI-1 therapy through antibody engineering″ (defended on 25th June 2009).

Contributions of authors:

PJD and AG were responsible for the generation of a panel of MA towards a stable human TAFI variant and for designing research, interpreting the data and reviewing the manuscript. EV and RB screened the panel of MA and selected MA with special features. Characterization of MA-TCK27A4 was performed by JD. NM characterized MA-TCK11A9 and MA-TCK22G2, analysed and interpretated the data, performed statistical analysis and drafted the manuscript.

CHAPTER 4

The Relative Contribution Of The Different TAFI Activators Regulating Fibrinolysis

Niraj Mishra, Paul J. Declerck, Ann Gils

Laboratory for Therapeutic and Diagnostic Antibodies,

Faculty of Pharmaceutical Sciences,

Katholieke Universiteit Leuven, Leuven

Manuscript in preparation

4.1. Abstract

Background: Activated thrombin activatable fibrinolysis inhibitor is an important regulator of t-PA-mediated fibrinolysis. Until now the thrombin/thrombomodulin (T/TM) complex was believed to be the physiological activator of thrombin activatable fibrinolysis inhibitor (TAFI), but recent *in vitro* and *in vivo* studies revealed that plasmin could also contribute to TAFI activation during fibrinolysis.

Objectives: To characterize TAFI variants selectively activatable by various activators to study the mechanism of TAFI activation during fibrinolysis.

Methods and results: In the current study, two TAFI variants previously reported to be selectively activatable by the T/TM complex (TAFI-K133A) or plasmin (TAFI-P91S) were evaluated in an *in vitro* clot lysis assay for their antifibrinolytic effects in TAFI-depleted plasma. The profibrinolytic effects of MA-T12D11 and MA-TCK26D6 which inhibit T/TM- and plasmin-mediated TAFI activation, respectively, were also evaluated in the presence of these variants. Both the mutants (TAFI-P91S and TAFI-K133A) revealed a 1.8- to 2-fold lower antifibrinolytic effect compared to TAFI wt. The profibrinolytic effect of MA-T12D11 in presence of TAFI wt was similar to the reduced antifibrinolytic activity observed for TAFI-K133A and TAFI-P91S. MA-T12D11 was able to completely abolish the effect of TAFI-K133A whereas MA-TCK26D6 was able to completely abolish antifibrinolytic effect of TAFI-P91S.

Conclusion: To the best of our knowledge this is the first detailed report revealing the relative contribution of the T/TM complex and plasmin in the regulation of fibrinolysis during *in vitro* clot lysis using selectively activatable TAFI variants and a combination of these variants with MA.

4.2. Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) is a zinc-dependent metallocarboxypeptidase, which is synthesized in the liver as a pre-proenzyme and secreted into the circulation as a highly glycosylated zymogen. Trypsin-like enzymes such as plasmin, thrombin or the thrombin–thrombomodulin (T/TM) complex proteolytically cleave TAFI at Arg^{92} resulting into the release of the glycosylated activation peptide and the generation of activated TAFI (TAFIa) [19, 25, 38]. The best studied function of TAFIa is its role in the regulation of fibrinolysis. During fibrinolysis, the C-terminal lysine residues on partially degraded fibrin act as a cofactor for the t-PA-plasminogen complex and consequently increase the catalytic efficiency of plasmin formation. TAFIa removes these newly exposed C-terminal lysines from partially degraded fibrin, impedes plasmin formation and attenuates fibrinolysis [176, 39]. Reports suggest that only a small amount of TAFI activation (TAFIa concentration; 1 nM, < 2% of the total plasma concentration of TAFI) is needed to downregulate the t-PA-mediated fibrinolysis [25, 38].

Thrombin is a weak activator of TAFI, but both the cellular and soluble forms of the endothelial cell receptor thrombomodulin enhance the activation of thrombin-mediated TAFI activation by 1250-fold [38, 41]. Plasmin is a stronger activator of TAFI than thrombin and the efficiency of plasmin-mediated activation is enhanced by glycosaminoglycans such as heparin [178]. However, the catalytic efficiency of the plasmin-heparin complex is still 10-fold lower compared to the T/TM complex [38, 178]. In literature, *in vitro* analyses describe the T/TM complex as the main physiological activator of TAFI [42, 43]. One *in vivo* study also demonstrated that TAFIa generated during *E.coli*-induced sepsis in baboons is inhibited by a monoclonal antibody (MA) specifically inhibiting T/TM-mediated activation of human TAFI [44]. However, recent *in vitro* characterization of MA/Nbs which inhibit TAFI activation by different mechanisms, suggests that next to T/TM-mediated TAFI activation, plasmin-mediated TAFI activation might also play an important role in the regulation of fibrinolysis [90, 154]. The contribution of the plasmin-mediated TAFI activation was further demonstrated using an *ex vivo* whole blood thrombi model and an *in vivo* mouse thromboembolism model [81, 179].

Recently, Miah *et al*. [43] and Vercauteren *et al*. [81] reported TAFI variants which are resistant to activation by either the T/TM complex or plasmin. Previously, monoclonal antibodies (MA) which inhibit TAFI activation through different mechanisms have been generated and characterized [81, 88, 154]. The present study was planned to evaluate the relative contribution of plasmin- and T/TM-mediated TAFI activation in the regulating of fibrinolysis using different TAFI variants selectively activatable by different TAFI activators.

4.3. Experimental procedures

4.3.1. Materials

All the experiments done in this study were performed with the wild-type form of human TAFI (TAFI-T^{147}-Ile^{325}, TAFI wt, without additional tags). Primers for site-directed mutagenesis and sequencing were purchased from Sigma-Aldrich (St Louis, MO, USA). *Pfx*50 DNA polymerase was obtained from Life Technologies (Gent, Belgium). Polymerase chain reactions were carried out with the Mastercycler-Gradient from Eppendorf (Hamburg, Germany). Restriction enzyme *Dpn*I was purchased from New England Biolabs (Hertfordshire, UK). Plasmid DNA purification was performed with the Plasmid mini kit I (Omega Bio-Tek, Doraville, GA, USA) and the Nucleobond™ AX500 kit (Machery-Nagel, Düren, Germany). DNA was sequenced by LGC Genomics (Berlin, Germany). Human thrombin, human plasmin, rabbit thrombomodulin and H-D-phenylalanyl-D-prolyl-L-arginine chloromethyl ketone (PPACK) were obtained from Sigma-Aldrich (St Louis, MO, USA), Enzyme Research Labs (South Bent, UK), American Diagnostica (Greenwich, CT, USA) and Biomol Research Labs (Plymouth Meeting, PA, USA), respectively. Hippuryl-L-arginine and aprotinin were bought from Bachem (Bubendorf, Switzerland) and Sigma-Aldrich (St Louis, MO, USA), respectively. Tissue-type plasminogen activator (t-PA, Actilyse®) was obtained from Boehringer Ingelheim. Dulbecco′s modified Eagles′ medium, OptiMEM-1 medium containing glutamax and, Lipofectamine™ 2000® were purchased from Invitrogen (Merelbeke, Belgium). X-tremeGene 9 was bought from Roche Diagnostics GmbH, Roche Applied Science (Mannheim, Germany). Horseradish peroxidase (HRP) type VI was purchased from Sigma- Aldrich.

4.3.2. Methods

4.3.2.1. Monoclonal antibodies

The monoclonal antibodies MA-T12D11, raised toward TAFI purified from plasma and mainly inhibiting T/TM-mediated TAFI activation [88] and MA-TCK26D6, raised toward the stable human TAFI variant TAFI-A147-C305-I325-I329-Y333-Q335 (TAFI-ACIIYQ) in TAFI-deficient mice, mainly inhibiting plasmin-mediated TAFI activation [81] were used throughout this study.

4.3.2.2. Preparation of TAFI depleted plasma

Citrated plasma of 33 healthy human individuals was collected in-house with their written consent and was pooled for preparation of TAFI-depleted plasma (TDP). TDP was obtained using MA-T4E3 coupled Sepharose 4B beads as described earlier [155].

4.3.2.3. Generation and production of TAFI variants

The cDNA of TAFI-TI was previously cloned into the pcDNA3.1(+) vector [33]. Site-directed mutagenesis (QuikChange, Stratagene) was performed to introduce the P91S mutation. The K133A TAFI variant was previously generated [81]. We expressed and purified TAFI wt, TAFI-P91S and TAFI-K133A as described previousely [33].

4.3.2.4. Evaluation of activatability of TAFI variants

The activatability of TAFI variants was measured as described earlier [90]. Purified TAFI [90 nM (final concentration) in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, + 0.1% BSA pH 7.4)] was incubated with plasmin (final concentration: 500 nM) in the presence of $CaCl_2$ (final concentration: 5 mM) for 10 min at 22 °C for TAFI activation. After activation, aprotinin (1.25 µM) and the substrate hippuryl-L-arginine (final concentration: 4 mM) was added to the activation mixture and substrate conversion was allowed to proceed for 30 min at 22 °C. Reactions (100 µL) were stopped by addition of 20 µL of 1N HCl followed by 20 µL of 1N NaOH. Then, 25 µL of 1M Na_2HPO_4 (pH 7.4) and 30 µL of 6% cyanuric chloride (dissolved in 1,4-dioxane) were added and the mixture was vortexed and centrifuged. 100 µL of the supernatant was transferred into a 96-well microtiter plate and the absorbance at 405 nm was measured. To be able to express the TAFIa activity in U mg^{-1}, a standard spanning 15.6 µM to 2 mM hippurate was used to quantify the amount of hippurate formed by TAFI [90, 154]. Within the concentration range used, a linear correlation between the generated TAFIa activity and colour development was observed. One unit (U) carboxypeptidase activity is defined as the amount of enzyme converting 1 micromole of substrate into product per minute at 37 °C.

Evaluation of the effect of the T/TM complex on TAFI activation was performed as described above, with some modifications. Thrombin (final concentration: 20 nM), and thrombomodulin (final concentration: 5 nM) was used instead of plasmin and aprotinin was replaced by PPACK (final concentration: 37.5 µM) to stop T/TM-mediated activation. Substrate conversion was allowed to proceed for 10 min at 22 °C instead of 30 min. The effect of the T/TM complex and plasmin on the activation of TAFI-variants to TAFIa was also evaluated on SDS-PAGE as described earlier [154].

4.3.2.5. Evaluation of the effect of various TAFI variants in clot lysis assay

To evaluate the effect of the various TAFI activators *in vitro* clot lysis experiments were designed using TDP reconstituted with either TAFI wt or one of the generated TAFI mutants TAFI-P91S and TAFI-K133A in the presence and absence of antibodies MA-T12D11 and MA-TCK26D6. Clot lysis was performed in a 96-well microtiter plate as described previously [154] with some modifications. Pooled TDP (final concentration: 30%) was incubated with either dilution buffer (10 mM Tris, 0.01%tween 20; pH 7.5) or with TAFI variants (final concentration: 54 nM), and/or antibodies (final concentration: 432 nM, resulting in an 8-fold molar excess over TAFI). The plate was incubated at 37 °C for 10 min and t-PA (final concentration: 120pM) was added. Then clot formation was induced by the addition of $CaCl_2$ (final concentration: 10.6 mM) and the plate was read at 2-min intervals at 405 nm at 37 °C. The 50% clot lysis time was determined and defined as the time interval between the time point of full clot formation to the midpoint of the maximum turbidity to clear transition.

4.3.2.6. Crystallographic structural representation

The structure of TAFI was taken from the protein databank (PDB ID: 3D66) [55] and represented using Pymol (DeLano Scientific LLC, Palo Alto, USA) (Fig. 4.1).

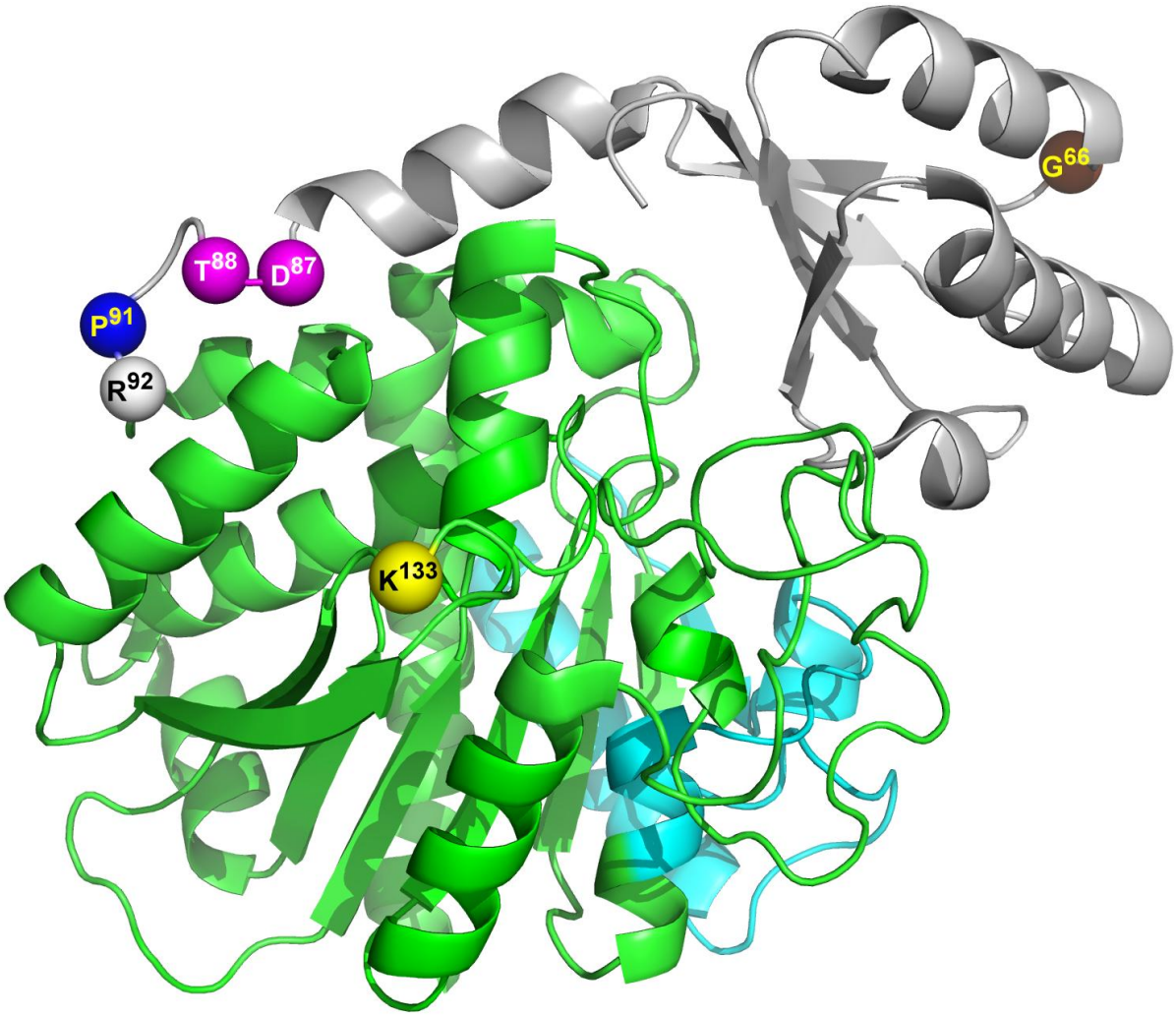

Figure 4.1. Ribbon diagram representing the 3D structure of TAFI. Grey, green and cyan colors represent the activation peptide, the catalytic domain and the dynamic flap of TAFI, respectively. R^{92} (white residue) is the cleavage site for plasmin, thrombin and the T/TM complex. Residues responsible for selective activation of TAFI by plasmin (P^{91}) and the T/TM complex (K^{133}) are represented in blue and yellow colored spheres, respectively. The epitope of MA-T12D11 (G^{66}) and MA-TCK26D6 (D^{87} and T^{88}) are depicted in brown and pink colored spheres, respectively.

4.3.2.7. Statistical analysis

Quantitative data were summarized by mean and standard deviation obtained from at least three independent experiments. Statistical significance was determined with the unpaired t-test using Graph Pad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). Values were considered as statistically significant at two-tailed p-values < 0.05. For the clot lysis experiments a paired two-tailed t-test was performed.

4.4. Results

4.4.1. Characterization of TAFI variants

To generate a mutant which is selectively activatable by plasmin the P91S mutation was introduced [43]. TAFI-K133A, selectively activatable by the T/TM complex has previously been generated in our laboratory [81]. Residue 91 is located near the cleavage bond whereas residue 133 is located in the catalytic site. Purified recombinant TAFI variants were tested for TAFIa activity upon activation by either plasmin or the T/TM complex. TAFIa activity generated through activation of TAFI wt with either plasmin or the T/TM complex was 9.40 ± 1.13 U mg^{-1} and 17.8 ± 2.07 U mg^{-1}, respectively (Table 4.1). Compared to TAFI wt, TAFI-K133A is 20-fold less activatable by plasmin and 1.4-fold more activatable by the T/TM complex whereas TAFI-P91S is 1.2-fold more activatable by plasmin and 9-fold less activatable by the T/TM complex.

Table 4.1: Activatability of TAFI variants by plasmin and the T/TM complex

TAFI-variant	TAFI-TI variants activation (U/mg)	
	By plasmin	By T/TM
TAFI-TI (wt)	9.40 ± 1.13	17.8 ± 2.07
TAFI-TI-P91S	10.9 ± 1.58	2.09 ± 1.10*
TAFI-TI-K133A	0.47 ± 0.23*	24.7 ± 4.65*

Mean ± SD, $n \geq 3$; * $p < 0.01$ vs. TAFI.

To confirm the effect of TAFI activators on different TAFI variants, fragmentation products of TAFI upon activation of TAFI variants by plasmin or the T/TM complex were analyzed using SDS-PAGE. These data confirmed that TAFI-K133A is selectively activatable by the T/TM complex and TAFI-P91S is selectively activatable by plasmin (data not shown).

4.4.2. Effect of TAFI variants during *in vitro* clot lysis assay

In order to investigate the functional effect of the different TAFI variants on clot lysis assay, TDP was preincubated either with buffer (negative control) or TAFI variants. Calcium was used to initiate coagulation and 120pM t-PA (final concentration) was used to initiate fibrinolysis. The 50% clot lysis time in TDP was 44 ± 12 min (Table 4.2 and Fig. 4.2). Addition of TAFI wt in TDP prolonged clot lysis time 4.3-fold (184 ± 34 min) whereas TAFI-P91S and TAFI-K133A were only able to prolong clot lysis time 2.4-fold and 2.1-fold (103 ± 24 min and 93 ± 23 min vs. 44 ± 12 min for no addition), respectively in TDP.

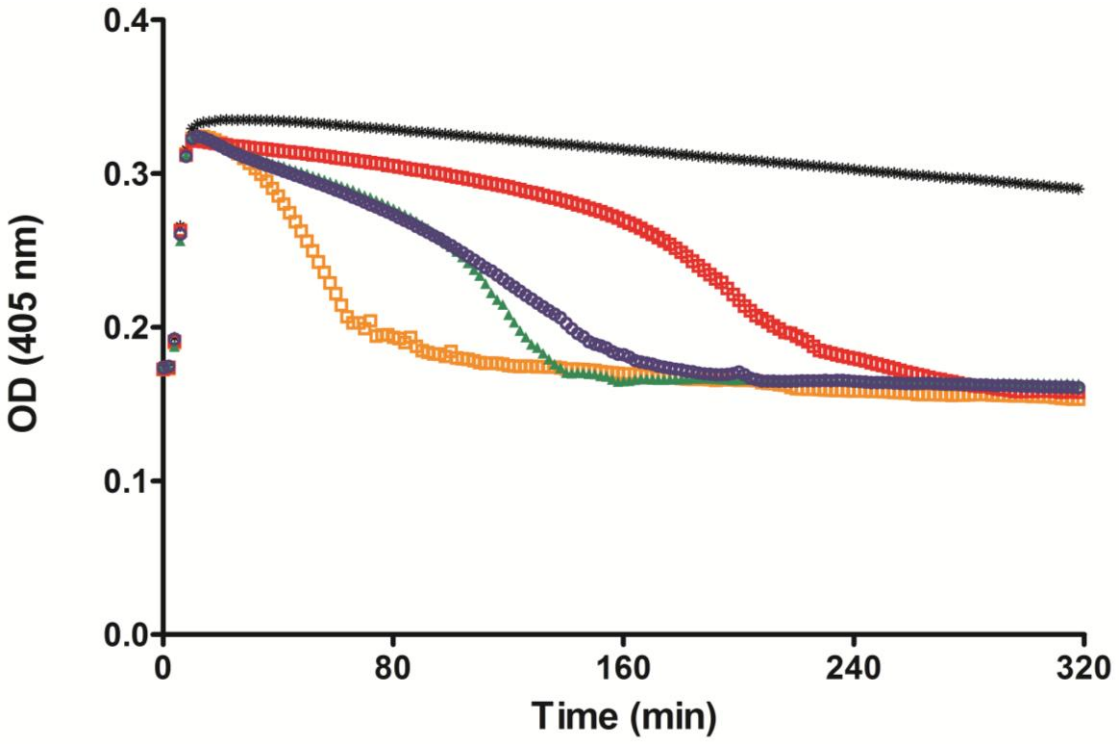

Figure 4.2. Clot lysis profile of TAFI variants. Clot lysis profile in TDP supplemented with TAFI wt (□), TAFI-P91S (○), TAFI-K133A (▲) and TDP alone (□) with 120pM t-PA and TDP alone without 120pM t-PA (∗). The representative curves are shown.

4.4.3. Effect of TAFI variants in presence of MA-T12D11 and MA-TCK26D6 during *in vitro* clot lysis

MA-T12D11 mainly inhibits T/TM-mediated TAFI activation [88]. Addition of MA-T12D11 to TDP supplemented with either TAFI wt, TAFI-P91S, or TAFI-K133A reduced clot lysis time 2.2-, 2.3- and 3.7-fold, respectively (Table 4.2). MA-TCK26D6 mainly inhibits plasmin-mediated TAFI activation [81]. Addition of MA-TCK26D6 to TDP supplemented with either TAFI wt, TAFI-P91S or TAFI-K133A reduced clot lysis time 4.1-, 4.3- and 2.3-fold, respectively (Table 4.2).

Table 4.2: Effect of MA on 50% clot lysis time in presence of TAFI variants in TDP

Addition	t-PA (120pM)	
	CLT (min)	**Relative reduction in clot lysis time**
TAFI-TI (wt)	184 ± 34	1.00
TAFI-TI + T12D11	84 ± 17	2.20 ± 0.13*
TAFI-TI + TCK26D6	45 ± 9	4.09 ± 0.44*
TAFI-TI-P91S	103 ± 24	1.79 ± 0.08 *
TAFI-TI-P91S + T12D11	79 ± 17	2.33 ± 0.05*#
TAFI-TI-P91S + TCK26D6	43 ± 8	4.32 ± 0.24*#
TAFI-TI-K133A	93 ± 23	1.99 ± 0.14*
TAFI-TI-K133A + T12D11	51 ± 15	3.67 ± 0.59*†
TAFI-TI-K133A + TCK26D6	83 ± 25	2.28 ± 0.33*

Mean ± SD, n ≥ 3; ∗ p < 0.02 vs. TAFI-TI; # p < 0.01 vs. TAFI-TI-P91S and †p < 0.005 vs. TAFI-TI-K133A. CLT, clot lysis time, Relative reduction in clot lysis time *vs.* TAFI-TI

4.5. Discussion

TAFIa exerts an antifibrinolytic effect by removing C-terminal lysines from partially degraded FDPs and thereby abolishes their cofactor function in the activation of plasminogen by t-PA [19, 25, 38]. Until recently it was believed that the T/TM complex is the only activator of TAFI during fibrinolysis [42, 43], but recent *in vitro* and *in vivo* studies suggest that the plasmin and the T/TM complex both contribute to the TAFI activation in the regulation of fibrinolysis [81, 90, 154, 179]. TAFI variants selectively activatable by various activators (thrombin, the T/TM complex and/or plasmin) might be excellent tools to study the mechanism of TAFI activation, regulation and its role in physiological conditions. Therefore, TAFI mutants selectively activatable by either plasmin (TAFI-TI-P91S) [43] or the T/TM complex (TAFI-TI-K133A) [81] were included in the study.

Compared to TAFI wt, TAFI-K133A is 20-fold less activatable by plasmin and 1.4 fold more activatable by the T/TM complex whereas TAFI-P91S is 9-fold less activatable by the T/TM complex and 1.2-fold more activatable by plasmin.

The *in vitro* clot lysis assays performed in TDP supplemented with TAFI variants in this study revealed that TAFI-P91S and TAFI-K133A were only able to exert approximately half of the antifibrinolytic effect compared to TAFI wt (Table 4.2 and Fig. 4.2). A similar type of study was performed by Miah *et al*. [43] using TAFI wt and TAFI-P91S variant supplemented to TDP. Miah *et al*. concluded that plasmin-mediated TAFI activation is not important in the regulation of fibrinolysis. However, in their clot lysis assays they not only substituted 30% TDP with different concentrations of TAFI variants and $CaCl_2$ (10 mM, final concentration), but also supplemented with exogenous thrombin (6 nM vs. none, final concentration), t-PA (200 pm *vs.* 120pM, final concentration) and 75% phosphatidylserine/25% phosphatidylcholine vesicles (20 µM *vs.* none, final concentration). Therefore, the contradiction in the results might be attributed to the different experimental conditions used in both the assays. Substition of activated TAFI wt, TAFI-P91S and TAFI-K133A in TDP revealed that both the mutants have similar antifibrinolytic activity compared to TAFI wt (data not shown). This indicates that plasmin and the T/TM complex both are involved in TAFI activation during fibrinolysis.

To further confirm our results we also evaluated the effect of TAFI variants in presence of MA-T12D11 and MA-TCK26D6 which mainly inhibits T/TM- and plasmin-mediated TAFI activation, respectively, during *in vitro* clot lysis. Addition of MA-T12D11 and MA-TCK26D6 to TDP supplemented with TAFI-K133A and TAFI-P91S, respectively showed a synergistic effect on the reduction of clot lysis time (Table 4.2). Moreover, the reduced clot lysis time was found to be equivalent to the clot lysis time of TDP. MA-T12D11 also revealed a similar clot lysis profile in TDP supplemented with TAFI wt and TAFI-P91S. To our surprise, addition of MA-TCK26D6 completely abolished the TAFIa effect. A similar profibrinolytic effect of MA-TCK26D6 was previously demonstrated by Vercauteren *et al*. [81] using TAFI wt and TAFI-K133A.

To conclude, this is the first detailed report revealing the relative contribution of the T/TM complex and plasmin in the regulation of fibrinolysis using TAFI variants and a combination of TAFI

variants and MA reconfirming that plasmin-mediated TAFI activation also plays an important role in clot lysis and consequently in the regulation of fibrinolysis

Contributions of authors:

NM designed and performed research, analyzed and interpreted the data, performed statistical analysis and drafted the manuscript. PJD and AG designed research, interpreted the data and reviewed the manuscript.

CHAPTER 5

In Vitro Evaluation Of The Profibrinolytic Properties of Anti-TAFI Monoclonal Antibodies Using Human TAFI-transgenic Mice

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Niraj Mishra, Joost C.M. Meijers, Paul J. Declerck, Ann Gils

Laboratory for Therapeutic and Diagnostic Antibodies,

Faculty of Pharmaceutical Sciences,

Katholieke Universiteit Leuven, Leuven

(Submitted)

5.1. Abstract

Background: Activated thrombin-activatable fibrinolysis inhibitor is an important regulator of t-PA-mediated fibrinolysis in humans as well as mice. Recently, TAFI single-gene-deficient mice expressing human TAFI (hTAFI) have been generated. These transgenic hTAFI mice constitute an interesting tool to study the effect of TAFI inhibitors which selectively inhibit human TAFI.

Objectives: To establish a model to evaluate the inhibitory effect of anti-human TAFI monoclonal antibodies (MA) which inhibit the activation of human TAFI through different mechanisms.

Methods and results: Using an *in vivo* thromboembolism model in which we quantified fibrin deposition before and after injection of tissue factor (TF), we concluded that TF-induced fibrin deposition in hTAFI mice was much lower compared to WT mice (fibrin deposition in lungs after TF injection was 4.7, 28.1 and 84.7 µg/ml in TAFI KO, hTAFI and WT mice, respectively). Using *in vitro* thromboelastometry we were able to define conditions in whole blood i.e. 8 nM t-PA to induce clot lysis and evaluation of lysis 60 min after clot formation (L_{60}) that allowed quantitative evaluation of the effect of human TAFI inhibitors. Under these conditions, all inhibitory anti-human TAFI MA significantly enhanced fibrinolysis in blood from hTAFI mice (L_{60}; 50 ± 9%, 53 ± 9% and 78 ± 4% for MA-T12D11, MA-TCK11A9, MA-TCK27A4, respectively, *vs.* 8 ± 2% in the presence of a control MA; $p \leq 0.0001$).

Conclusion: We established an *in vitro* model which can be used to evaluate human TAFI inhibitors. Using this model we determined the profibrinolytic properties of a panel of MA that inhibit human TAFI activation through different mode of actions.

5.2. Introduction

The fibrinolytic system plays an important role in blood clot dissolution to preserve the blood fluidity within the vascular system. During fibrinolysis, inactive plasminogen is converted into active plasmin by tissue-type plasminogen activator (t-PA). Active plasmin then degrades fibrin into fibrin degradation products (FDPs) resulting in blood clot dissolution. Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) retards fibrinolysis by removing C-terminal lysine residues from partially degraded fibrin and thereby attenuates its cofactor function in the t-PA mediated plasmin generation [38, 164]. *In vivo* evidence for a role of TAFI in fibrinolysis have been obtained in several animal models through pharmacological inhibition [44, 78, 81, 95, 97, 102] as well as using TAFI single-gene-deficient mice (TAFI KO) [60, 83].

TAFIa is a metallocarboxypeptidase, which is synthesized in the liver and secreted into the circulation as a 56 kDa glycosylated zymogen. The proenzyme TAFI is cleaved at Arg^{92} by trypsin-like enzymes such as plasmin, thrombin or the thrombin/thrombomodulin (T/TM) complex, generating TAFIa [20]. Even though the T/TM complex is accepted to be the physiological activator of TAFI in the regulation of fibrinolysis [42, 43], recent *in vitro* and *ex vivo* studies suggest that plasmin also plays an important role in TAFI activation and consequently in the regulation of fibrinolysis [81, 154, 179]. However, *in vivo* studies are necessary to unravel the relative contribution of the different TAFI activators. To the best of our knowledge, only two studies reported inhibition of TAFIa generation *in vivo*. One *in vivo* study demonstrated that TAFIa generated during *E.coli*-induced sepsis in baboons is inhibited by a monoclonal antibody (MA) specifically inhibiting T/TM-mediated activation of human TAFI [44]. A second study using a specific MA that mainly inhibits plasmin-mediated TAFI activation demonstrated a strong profibrinolytic effect in a mouse thromboembolism model [81].

To date, no physiological inhibitor of TAFIa is known [28]. However, TAFIa is sensitive to inhibition by chelating agents, by compounds disrupting disulfide bridges, by small synthetic substrate analog inhibitors and by naturally occurring metallocarboxypeptidase inhibitors [54, 62, 85, 87, 163]. Despite of an increasing list of TAFI inhibitors, the applicability of most of these inhibitors is restricted given their lack in selectivity, safety and the occurrence of a biphasic effect. On the other hand monoclonal antibodies (MA) have been established as candidate drug molecules with a high specificity and stability. MA that hamper plasmin-, thrombin- and T/TM-mediated activation of TAFI to TAFIa as well as MA that inhibit TAFIa activity directly have been described [30, 44, 81, 88, 89, 154, 164]. Unfortunately, most of these MA reveal no cross-reactivity with mouse or with rat TAFI, thereby excluding their *in vivo* evaluation in small animals [88, 154].

The amino acid sequence of mouse and rat TAFI share approximately 85% identity with human TAFI including conservation of important residues involved in substrate binding and specificity, glycosylation and zinc binding. TAFI from all three species have similar biochemical characteristics with respect to their activatability by the T/TM complex and their antifibrinolytic effect during *in vitro* clot lysis [34-36]. Addition of human TAFI was reported to be more efficient in retarding clot lysis in mouse and rat TAFI-depleted plasma compared to mice and rat TAFI [35]. Recently, mice expressing

human TAFI (hTAFI) were generated from TAFI KO mice [181]. These mice might be an excellent tool to evaluate the effect of TAFI inhibitors exclusively inhibiting human TAFI.

In the present study, we aimed to evaluate the use of human TAFI transgenic mice to confirm the profibrinolytic properties of anti-human TAFI MA.

5.3. Experimental procedures

5.3.1. Animals

C57BL/6J mice (WT) were purchased from Charles River Laboratories (Wilmington, MA, USA). TAFI KO mice (in a 100% C57BL/6J background) and TAFI KO mice in which the cDNA encoding human TAFI was transferred (transgenic hTAFI-mice in a 100% C57BL/6J background) were generated as previously described [60, 181]. Mice were kept in microisolation cages on a 12-h day–night cycle with water and food ad libitum. Housing and procedures involving experimental animals were conducted in accordance with institutional guidelines and on a license (P087/2011) approved by the Ethical Committee of the KU Leuven, Belgium. All thromboembolism experiments were carried out with 6-8 weeks old male mice (weighing 20 to 25 g; 8 TAFI KO, 43 hTAFI and 33 WT mice in total) and all rotational thromboelastometry (ROTEM) analyses were carried out with 12-16 weeks old male mice (9 TAFI KO, 17 hTAFI and 9 WT mice in total)

5.3.2. Methods

5.3.2.1. Genotypic analyses

Genomic DNA was isolated from mouse tail biopsies as previously described [78] and used as a template in PCR to verify the genotypes. Each sample from hTAFI mice and TAFI KO mice were subjected to two separate PCR reactions with the following primer sets to detect (i) the human TAFI gene, i.e. forward primer 5'- CTGTGAGCAGCATGTCTTCG-3' and reverse primer 5'-CACAAGCCTCAGGAGATTGG-3', respectively), detecting a 601-bp amplification product in hTAFI+/+ and hTAFI+/-) samples and (ii) the mouse TAFI gene, i.e. forward primer 5'-GCTCTGGTTCTCTGGTTGG-3' and reverse primer 5'-CAGTCTTCTATGGTAACAGC-3' (annealing in introns 1 and 2 [ENSMUSG00000021999]) detecting a 446-bp amplification product in $TAFI^{+/+}$ and $TAFI^{+/-}$ samples or reverse primer 5'-TATTGCTGAAGAGCTTGGCGGCG-3' (derived from the phosphoglycerine thymidine kinase–neomycin expression cassette, pKONEO) detecting a 574-bp amplification product in $TAFI^{+/-}$ and $TAFI^{-/-}$ sample. PCR products were electrophoresed on a 1.5% agarose gel, post-stained with 2 X GelRed (Biotium, Hayward, CA, USA) in 1 M NaCl, and visualized with a UV transilluminator and the UVP Biodoc-It Imaging System (UVP, Upland, CA, USA).

5.3.2.2. Determination of human TAFI and mouse TAFI antigen levels in hTAFI mouse

Following anesthesia (isoflurane; 2.5%), blood was collected via the retro-orbital vein on sodium citrate 3.8% (1:10 v/v). Plasma was prepared by centrifugation of blood at 2000 × *g* for 20 min. TAFI antigen levels in mouse plasma were determined using in-house developed ELISAs, MA-RT36A3F5/MA-RT82F12-HRP [182] and MA-T12D11/pAb-anti-TAFI-HRP, for mouse and human TAFI, respectively.

5.3.2.3. Monoclonal antibodies

Monoclonal antibody MA-T12D11, mainly inhibiting T/TM-mediated TAFI activation was raised toward the plasma-derived human TAFI [88]. MA-TCK11A9, mainly inhibiting plasmin-mediated TAFI activation [81, 154] and MA-TCK27A4, inhibiting plasmin-, thrombin- and T/TM-mediated TAFI

activation [154] were raised toward the stable human variant TAFI-A^{147}-C^{305}-I^{325}-I^{329}-Y^{333}-Q^{335} (TAFI-ACIIYQ) [52]. All these MA only react with human TAFI. MA-Tom1-41B2 directed toward tomato pectin methylesterase [183] reveals no cross-reactivity with human nor with mouse TAFI and was used as negative control MA throughout this study.

5.3.2.4. *In vitro* rotational thromboelastometry (ROTEM) analysis

Blood samples of the three genotypes (TAFI KO, hTAFI and WT) were collected by retro-orbital vein puncture into sodium citrate (3.8%, 1:10 v/v), and analyzed on a ROTEM delta instrument (Tem International, Munich, Germany) as described previously, with some modifications [78]. The blood samples (300 µL) were incubated with $CaCl_2$ (final concentration: 20 mM) and increasing doses of recombinant human t-PA (final concentration: 4–20 nM; Actilyse; Boehringer Ingelheim, Brussels, Belgium). The clot formation and lysis reaction was monitored for 2 h. The degree of lysis (L,%) defined as the decrease in amplitude (mm) relative to the maximal amplitude ($[(A_{max}-A_t)/A_{max}]*100$) was determined at 30 min, 45 min (L_{45}), 60 min (L_{60}) and 120 min after start of clot formation. The effect of the MA (328 nM; corresponding to 8-fold molar excess over TAFI compared to the concentration of human TAFI in male hTAFI mice) was evaluated in TAFI KO, hTAFI and WT mice in the presence of 8 nM t-PA. When no MA was added, PBS was added to the reaction mixture.

5.3.2.5. *In vivo* mouse thromboembolism model

The mouse thromboembolism model and fibrin extraction was performed as described previously [81]. Briefly, saline was injected intravenously in the tail vein of non-anesthetized TAFI KO, hTAFI and WT mice. After 5 minutes, thromboplastin (2.5 µg/kg, intravenously) was administered to induce thromboembolism in the lungs. Control mice only received saline injections. Mice were anesthetized with pentobarbital (Nembutal; 60 mg/kg intraperitoneally) 10 minutes after saline/thromboplastin injections. To prevent postmortem coagulation, heparin (heparin LEO; 500 IU) was injected in the vena cava 20 minutes after thromboembolism induction. Three minutes later, lungs were perfused with saline containing heparin (10 IU/mL). Isolated left and right lungs were snap-frozen in liquid nitrogen and stored at (-80°C) until homogenization. For homogenization, 4 mL of PBS per gram of tissue was added to the frozen lungs. Subsequently, the lung tissues were homogenized with a tissue homogenizer (Ribolyzer Fast Prep 24 System, MP Biomedicals) and centrifuged. After resuspension of the pellet containing insoluble fibrin, ocriplasmin (final concentration: 2µM) (a kind gift from Thrombogenics, Leuven, Belgium) was added to convert insoluble fibrin into soluble FDPs. Subsequent to incubation of the samples for 4 hours at 37°C, the samples were centrifuged. The supernatants were collected and fibrinolysis was stopped by adding aprotinin (final concentration: 4µM). FDPs and thereby the corresponding fibrin deposition was determined using a commercial ‘‘Mouse Fibrinogen ELISA” with reactivity for FDPs (Immunology Consultants Laboratory, Portland, USA) as per manufacturer’s instruction. Data represent the average of the fibrin deposition in left and right lungs.

5.3.2.6. Statistical analysis

Quantitative data were summarized by mean and standard error of mean (SEM) obtained from at least three independent experiments except for human TAFI levels and EC_{50} of t-PA which were summarized as mean and standard deviation (SD). Statistical significance was determined with the unpaired t-test using Graph Pad Prism 5.03 (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered as statistically significant at two-tailed p-values < 0.05. A nonlineair regression (log agonist *vs.* normalized response-variable slope) was used to calculate the EC_{50} of t-PA. D'Agostino & Pearson omnibus normality test was performed to analyze distribution of human TAFI antigen in the plasma of male and female hTAFI mice.

5.4. Results

5.4.1. Genotypic analysis and TAFI plasma levels in hTAFI mouse

Genotypic analyses confirmed that hTAFI mice only carry the human TAFI gene and lack the mouse TAFI gene [181]. Mouse and human TAFI protein levels were determined in plasma of hTAFI mice. No mouse TAFI was detected in plasma of hTAFI mice (i.e. < 0.2 µg/ml) whereas human TAFI was expressed at 1.8 ± 1.2 µg/ml (average of male and female mice); range: 0.2-4.9 µg/ml; n = 237. A 3.5-fold higher level was observed in male *vs.* female hTAFI mice (2.6 ± 0.9 µg/ml; range: 0.9-4.9 µg/ml; n = 133 *vs.* 0.72 ± 0.36 µg/ml; range: 0.2-1.9 µg/ml n = 104, respectively; $p < 0.0001$). Our distribution analyses for the plasma TAFI concentration revealed that data were not normally distributed in both female ($p = 0.002$) as well as in male ($p = 0.043$) hTAFI mice.

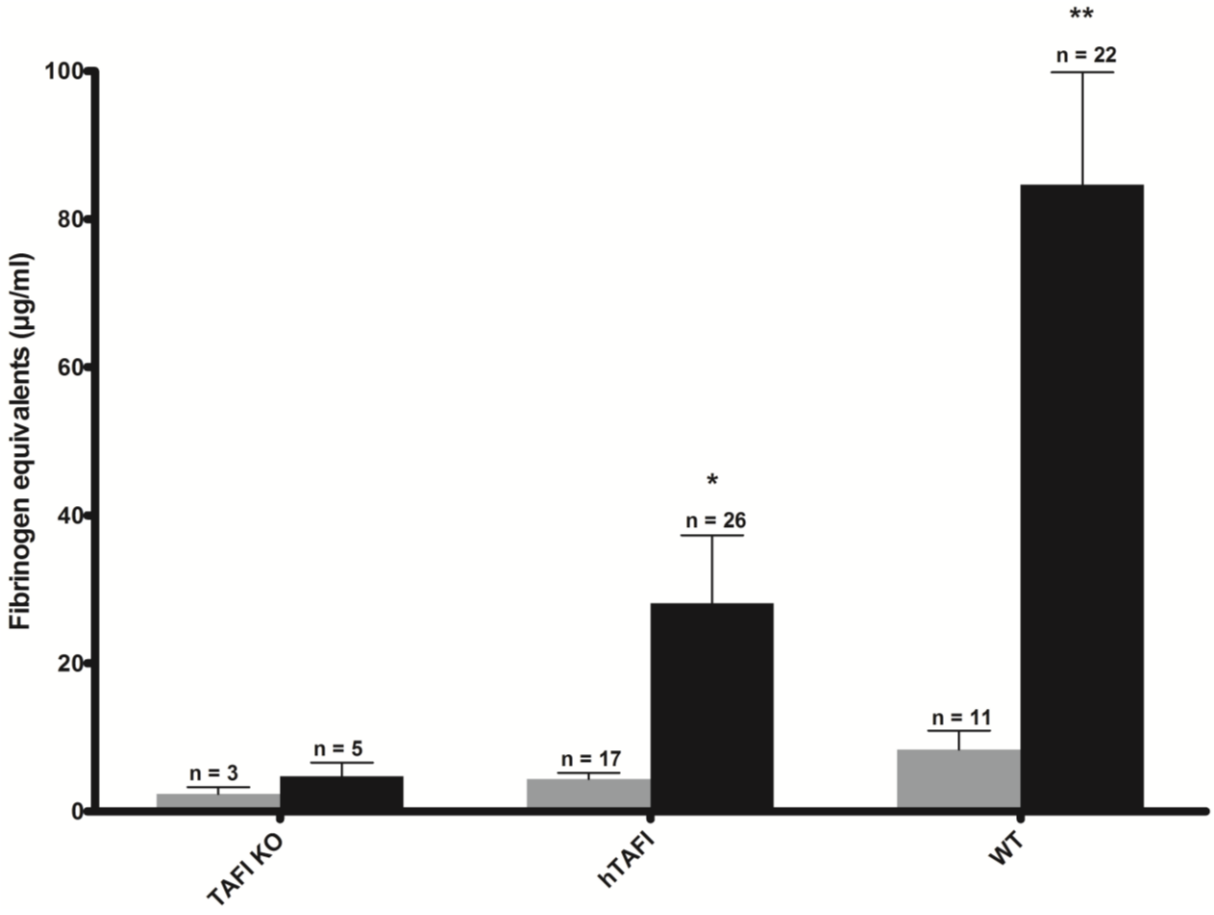

Figure 5.1: Fibrin deposition in lungs before (grey bars) and after (black bars) induction of thromboembolism. FDPs are expressed as µg/ml fibrinogen equivalents and are represented as mean ± SEM. Significant differences (after *vs.* before thromboembolism) are indicated (* $p < 0.05$ and ** $p < 0.002$).

5.4.2. Thromboembolism model

To quantify the fibrinolytic capacity of hTAFI mice, fibrin deposition was quantified in the lungs of male TAFI KO, hTAFI and WT mice before and after induction of thromboembolism (Fig. 5.1). Before injection of thromboplastin, TAFI KO, hTAFI and WT TAFI mice revealed very low fibrin deposition in lungs (2.3 ± 0.9 µg/ml, 4.4 ± 0.8 µg/ml, 8.3 ± 2.6 µg/ml, respectively). No significant difference was

observed among the three genotypes before injection of thromboplastin. Upon injection of thromboplastin, deposition of fibrin did not significantly increase in TAFI KO mice (4.7 ± 1.8 µg/ml n = 5 *vs.* 2.3 ± 0.9 µg/ml n = 3, p > 0.05) but significantly increased in WT mice (84.7 ± 15.1 µg/ml n = 22 *vs.* 8.3 ± 2.6 µg/ml n = 11, p < 0.002) as previously described by Vercauteren *et al*. [81]. Even though in hTAFI mice a significant increase in fibrin deposition was observed in the lungs upon tissue factor injection (28.1 ± 9.2 µg/ml n = 26 *vs.* 4.4 ± 0.8 µg/ml n = 17, p < 0.05), the intrinsic high variability precluded the use of this thromboembolism model in hTAFI mice for reliable evaluation of TAFI inhibitors that react with human TAFI.

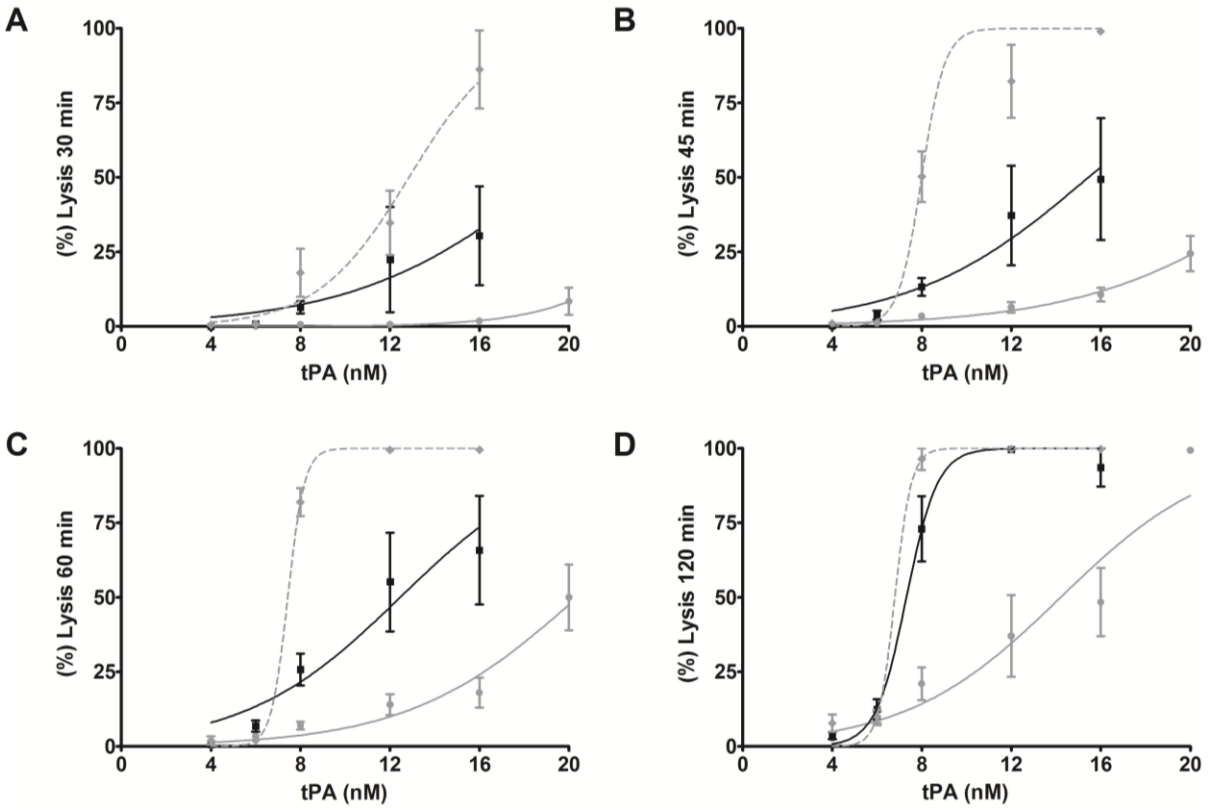

Figure 5.2. Lysis in rotational thromboelastometry with increasing tissue-type plasminogen activator (t-PA) concentration. Lysis (%) is expressed as the decrease in amplitude (mm) relative to the maximal amplitude ([(Amax-At)/Amax]*100) at 30 min (A), 45 min (B), 60 min (C) and 120 min (D). Blood from TAFI KO mice (grey curve; --◆--) human TAFI-transgenic mice (black curve; –■–) and WT mice (solid grey curve; –●–). Data represent mean ± SEM, n = 5 for each set of experiments.

5.4.3. *In vitro* rotational thromboelastometry analysis

To explore the effect of human TAFI on fibrinolysis in mice, whole blood samples (n = 5) from TAFI KO, hTAFI and WT mice were analyzed by thromboelastometry using different t-PA concentrations (Fig. 5.2). Compared to lysis in blood from WT mice, lysis in hTAFI mice was accelerated at all the t-PA concentrations above 6 nM at any time point (30 min, 45 min, 60 min and 120 min, Fig. 5.2A, B, C and D, respectively). Compared to lysis in blood from TAFI KO mice, lysis was delayed in blood from hTAFI mice at 30 min, 45 min and 60 min, but this effect disappeared at 120 min (EC_{50} values for t-PA

at t=120 min: 6.8 ± 0.1 nM, 7.3 ± 0.2 nM and 14.2 ± 0.7 nM for TAFI KO, hTAFI and WT mice, respectively; $p < 0.0001$ TAFI KO and hTAFI *vs.* WT; Fig. 5.2D).

Using 8 nM t-PA, L_{45} was 50 ± 9% 13 ± 3% and 3 ± 1% for TAFI KO, hTAFI and WT mice, respectively ($p < 0.005$, $p < 0.001$ and $p < 0.01$ for TAFI KO *vs.* hTAFI, TAFI KO *vs.* WT and hTAFI *vs.* WT, respectively) and L_{60} was 82 ± 5% 26 ± 5% and 7 ± 1% for TAFI KO, hTAFI and WT mice, respectively ($p < 0.0001$, $p < 0.0001$ and $p < 0.01$ for TAFI KO *vs.* hTAFI, TAFI KO *vs.* WT and hTAFI *vs.* WT, respectively). Based upon these data it was concluded that the use of 8 nM t-PA concentration and evaluation of the lysis at 60 min provided the most sensitive conditions for evaluation of the profibrinolytic effect of TAFI inhibitors.

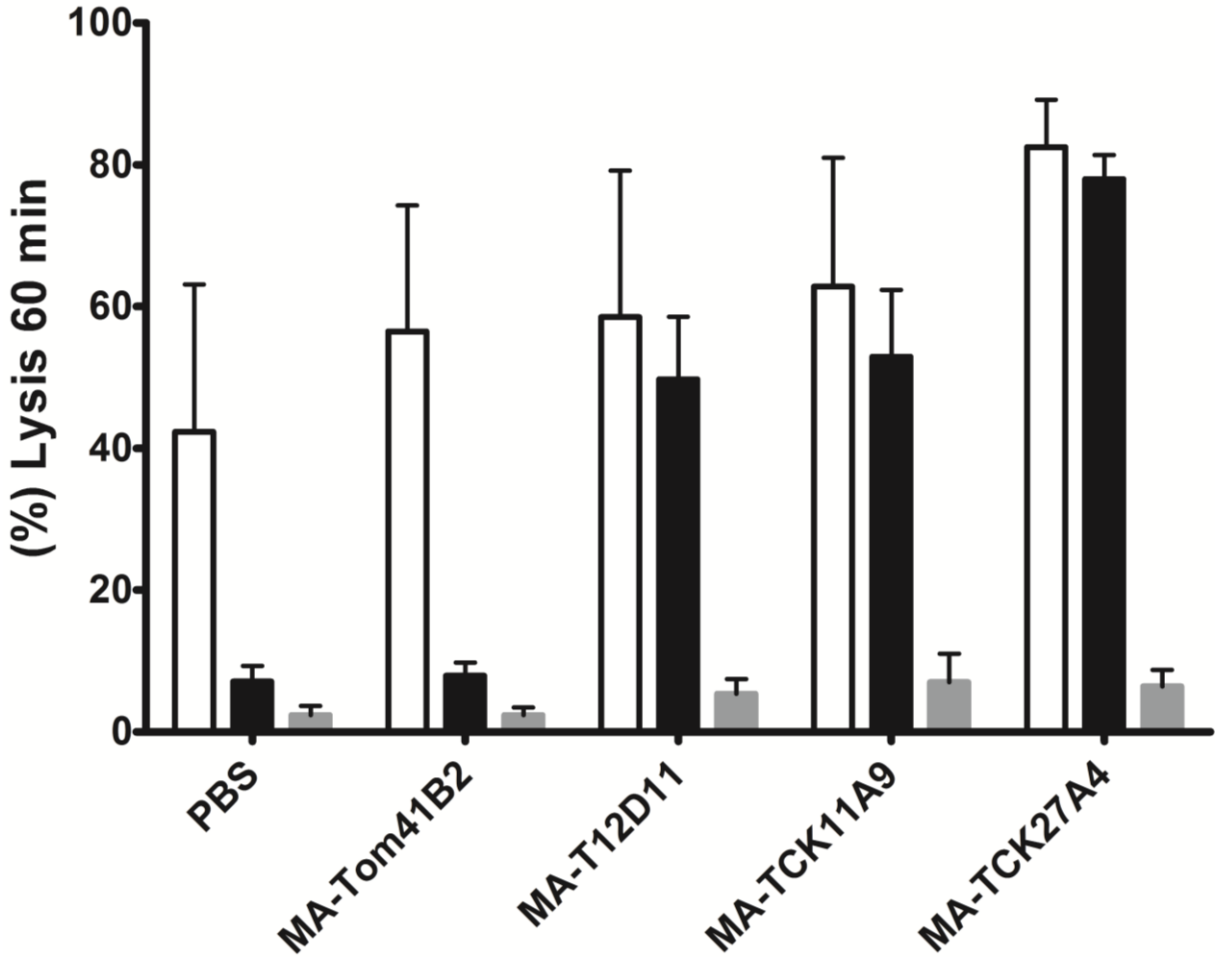

Figure 5.3: Effect of anti-human TAFI MA on t-PA-mediated fibrinolysis in rotational thromboelastometry. Lysis (%) is expressed as the decrease in amplitude (mm) relative to the maximal amplitude ([(Amax-At)/Amax]*100) at 60 min. Lysis in blood from TAFI KO mice (empty bars, n = 4), human TAFI mice (black bars, n = 12) and WT mice (grey bars, n = 4).

5.4.4. Effect of anti-human TAFI monoclonal antibodies on *in vitro* fibrinolysis

The effect of anti-human TAFI MA on *in vitro* fibrinolysis was evaluated by thromboelastometry in whole blood from TAFI KO (n = 4), hTAFI (n = 12) and WT mice (n = 4) using 8 nM t-PA concentration and evaluation of L_{60} (Fig. 5.3). The human-specific anti-TAFI MA and the control antibody MA-Tom1-41B2 did not affect fibrinolysis in WT mice (L_{60}; 2 ± 1%, 5 ± 2%, 7 ± 4% and 6 ± 2% for MA-Tom1-41B2, MA-T12D11, MA-TCK11A9 and MA-TCK27A4, respectively, *vs.* 2 ± 1% for PBS; $p > 0.05$ all *vs.*

PBS) and TAFI KO mice (L_{60}; 56 ± 18%, 58 ± 21%, 63 ± 18% and 83 ± 7% for MA-Tom1-41B2, MA-T12D11, MA-TCK11A9 and MA-TCK27A4, respectively, *vs.* 42 ± 21% for PBS; $p > 0.05$ all *vs.* PBS). However, in hTAFI mice all MA, except MA-Tom1-41B2 (L_{60} 8 ± 2%; $p > 0.05$ *vs.* PBS) significantly enhanced fibrinolysis (L_{60}, 50 ± 9%, 53 ± 9% and 78 ± 4% for MA-T12D11, MA-TCK11A9, MA-TCK27A4, respectively, *vs.* 8 ± 2% for MA-Tom1-41B2; $p < 0.0001$ all *vs.* MA-Tom1-41B2).

5.5. Discussion

Thromboembolism is one of the major causes of morbidity and mortality in the western world. Currently available therapy is associated with bleeding complications and requires close lab-monitoring [62]. Therefore, development of clinically safe and easy to use drugs is essential. Several *in vitro, ex vivo* and *in vivo* studies in various animal models, including the use of WT and TAFI KO mice, have indicated the important role of TAFI in the regulation of fibrinolysis [44, 60, 78, 81, 83, 95, 97, 102, 154, 179, 184]. Numerous clinical studies have also correlated TAFI antigen levels with cardiovascular events such as angina pectoris [168], coronary artery disease [169], ischaemic stroke [170], myocardial infarction [73] and venous thrombosis [69]. Considering the fact that TAFI plays a pivotal role in fibrinolysis while the coagulation cascade remains unaffected, TAFI targeting based therapies may result in fewer bleeding complications [62] and might also be useful in lowering the required dose of infused plasminogen activators during thrombolytic therapy. Alternatively, TAFI inhibitors could also be used in the prevention of thrombotic events. Since small molecule inhibitors of TAFIa revealed several drawbacks such as lack of selectivity and safety as well as a biphasic effect [85, 87], anti-human TAFI MA might be interesting lead molecules in the development of biotherapeutics to prevent and/or to treat thrombotic disorders. Recently MA have been generated against human TAFI that impair human TAFI activation in a highly selective manner through different mechanisms. Due to the fact that these MA do not cross react with mouse or rat TAFI, these MA cannot be evaluated *in vivo* in mice or rat models [88, 154].

In the present study we have characterized and evaluated hTAFI transgenic mice in models to determine the profibrinolytic properties of anti-human TAFI MA. First, we have determined the human TAFI antigen levels in hTAFI mice and observed that, for yet unknown reasons, male hTAFI mice express approximately 3.5-fold higher TAFI levels than female hTAFI mice (2.6 µg/ml *vs.*0.72 µg/ml, respectively). Therefore, we selected only male mice for further *in vitro* and *in vivo* experiments. The different genotypes (TAFI KO, hTAFI and WT mice) were subjected to a thromboembolism model by injection of tissue factor [81]. No significant differences were observed in fibrin deposition in lungs of TAFI KO mice before and after thromboplastin injection, whereas fibrin deposition in lungs of WT mice increased 10-fold after injection of thromboplastin. This is in accordance with previous studies [78, 81, 95, 97, 102] confirming the antifibrinolytic role of TAFI in the regulation of fibrinolysis. Even though fibrin deposition was also increased upon injection of thromboplastin in hTAFI mice, the 6-fold increase was less than that observed in WT mice. These data indicate that the hTAFI mice are less susceptible to fibrin deposition (or have a higher fibrinolytic capacity) than WT mice. Previous *in vitro* clot lysis studies in healthy individuals suggested that plasma TAFI levels influence the clot lysis time in a TAFIa dependent manner [31]. However, another study revealed that only a small amount of TAFI activation (TAFIa concentration; 1 nM, < 2% of the total plasma concentration of TAFI) is needed to downregulate the t-PA-mediated fibrinolysis and that rather than the concentration, the rate of TAFI activation and stability of TAFIa affects fibrinolysis [38]. In this study we did not found any correlation between human TAFI antigen levels (2.1 ± 0.7 µg/ml, range: 0.9-3.7 µg/ml; n = 22 in thromnoembolism-induced mice) and fibrin deposition in lungs of hTAFI mice (r; 0.1673 and P, 0.41).

The differences in fibrin deposition between hTAFI and WT mice could be result of a less efficient activation of human TAFI through the mouse thrombin-thrombomodulin complex or mouse plasmin. However, it is interesting to note however that once activated human TAFIa is 4-fold more stable than mouse TAFIa [35], thereby expecting rather a more pronounced antifibrinolytic effect of human TAFIa. Alternatively, the lower antifibrinolytic status observed in hTAFI mice could also result from a less efficient catalytic activity of human TAFIa on mouse fibrin. Indeed, TAFI activity in hTAFI mice was approx. 60% of TAFI activity in WT mice (data not shown). Inspite using twenty-six animals, we were only able to see a small significant TF-induced fibrin deposition in lungs of hTAFI mice ($p = 0.045$). The evaluation of TAFI inhibitors *in vivo* in hTAFI mice may provide some trend, but needs a large number of animals to evaluate multiple TAFI inhibitors which will be non-ethical and will be against the Russell and Burch 3R principle [185]. The lower susceptibility to fibrin deposition, the large intrinsic variability of the thromboembolism model and the non-ethical requirement of a large number of animals led us to conclude that the use of this model in hTAFI mice is not sensitive and suitable enough to study the effect of the TAFI inhibitors.

Previous studies revealed a TAFI-related retardation of t-PA-mediated fibrinolysis in rotational thromboelastometry [78, 186]. In the current study we performed comparative *in vitro* rotational thromboelastometry using whole blood from TAFI KO, hTAFI and WT mice. Evaluation of lysis at different time points using varying concentrations of t-PA revealed that, in line with the *in vivo* thromboembolism data, the fibrinolytic capacity of hTAFI mice (at L_{30}, L_{45} and L_{60}) is intermediate between that of TAFI KO and WT mice, but similar to TAFI KO mice 120 min after start of lysis (L_{120}). This might again be attributed to either less efficient TAFI activation or less efficient catalytic activity of human TAFIa on mouse fibrin. Since thromboelastometry appeared to be more sensitive and more flexible with respect to different conditions and parameters this model was further optimized for evaluation of the effect of human TAFI inhibitors in whole blood from hTAFI mice. All the three anti-human TAFI MA were found to exhibit strong profibrinolytic properties when tested on whole blood from hTAFI expressing mice. Compared to no addition and a negative control antibody MA-Tom1-41B2 (L_{60} = 7 to 8 %), MA-T12D11, MA-TCK11A9, and MA-TCK27A4 enhanced fibrinolysis up to ten-fold (L_{60} = 50 to 78 %). Under these conditions (L_{60} at 8 nM t-PA) none of the MA demonstrated any effect in TAFI KO mice or WT mice. These findings confirm the strong profibrinolytic effect of MA-T12D11, MA-TCK11A9, and MA-TCK27A4 towards human TAFI as previously demonstrated *in vitro* and also elucidated that whole blood from hTAFI mice constitute an excellent tool to evaluate TAFI inhibitors towards human TAFI (Fig. 5.3).

It is of interest to note that both MA-TCK11A9 which mainly prevents activation of human TAFI by plasmin and MA-T12D11, that inhibits the activation of human TAFI mainly by the T/TM complex, exert similar profibrinolytic properties in thromboelastometry (L_{60} = 50 to 53 %) indicating that both activators contribute to the activation of TAFI in the regulation of fibrinolysis in this whole blood thrombus model confirming previous reports [81, 154, 179]. MA-TCK27A4, inhibiting the activation of TAFI by plasmin, thrombin as well as the T/TM complex [154] showed a significantly higher lysis (L_{60} = 78 %; $p < 0.01$ *vs.* MA-T12D11) suggesting that the contribution of all TAFI activators is essential for the strongest antifibrinolytic effect. It should be taken into account however that in a recent study

(Semeraro *et al.* unpublished results) it was found that MA-TCK11A9 not only interferes with plasmin-mediated TAFI activation but might also reduce TAFIa activity in a functional assay using fibrin as a substrate. Therefore, further investigations are essential to explore the role of plasmin in the activation of TAFI.

This study demonstrates that hTAFI mice exhibit an intermediate fibrinolytic phenotype between TAFI KO and WT mice which corresponds with TAFI activity in blood.

The current study also demonstrates that the combination of rotational thromboelastometry and whole blood from hTAFI mice can be used as an alternative model to evaluate the *in vitro* effect of profibrinolytic compounds exclusively reacting with human TAFI. Moreover, the results confirm the strong *in vitro* profibrinolytic effect of inhibitory anti-human TAFI MA irrespective of their mechanism of inhibition of TAFI activation. Accordingly, it is also clear that enhancement of the t-PA-mediated fibrinolytic activity by impairing TAFI activation constitutes a promising pharmacologic approach to improve thrombolytic therapy.

Contributions of authors:

NM designed and performed research, analyzed and interpreted the data, performed statistical analysis and drafted the manuscript. JCMM provided hTAFI and TAFI KO mice and reviewed the manuscript. PJD and AG designed research, interpreted the data and reviewed the manuscript.

CHAPTER 6

Concluding Discussion

TAFI is the only metallocarboxypeptidase belonging to subfamily A (proenzymes) that demonstrates a zymogen activity towards small substrates. However, TAFI has an 18-fold lower catalytic efficiency compared to TAFIa [19, 54, 66].

Characterization of zymogen stimulating nanobodies towards TAFI

The crystal structure of human TAFI was found to be similar to that of pancreatic CPB which does not exhibit any zymogen activity. The structure also suggested that the catalytic site residues of TAFI readily remain in the active conformation and that activation is required only to expose the catalytic pocket and not to induce conformational changes at the active site [55, 187]. The absence of a salt bridge between the activation peptide and the catalytic domain in TAFI compared to pancreatic CPB provides a logical explanation for zymogen activity of TAFI. However, Foley *et al*. suggested that due to non-covalent interactions between the activation peptide (Val^{35} and Leu^{39}) and the catalytic domain (Tyr^{341}) only small substrates can reach the active site of TAFI. Therefore, TAFI zymogen can only cleave lysine and arginine residues from small substrates like hippuryl-arginine, but not from bigger substrates like partially degraded fibrin [40].

In our laboratory, we identified Nbs (Vhh-TAFI-a51 and Vhh-TAFI-i103) which can stimulate the zymogen activity of TAFI. Therefore, the first part of this thesis focuses on the *in vitro* characterization of zymogen stimulating Nbs (**Chapter 2**) to determine the role of ″stimulated″ zymogen activity of TAFI in the regulation of fibrinolysis. From our results we can deduce that binding of Nbs to TAFI might result in a translocation of the activation peptide, leading to an increased accessibility of the catalytic site. That is why the ″stimulated″ zymogen activity of TAFI was able to exert an antifibrinolytic effect. A previous study has demonstrated that an increased TAFIa activity restores the impaired antifibrinolytic potential in plasma of the patients with haemophilia A [188]. Therefore, these zymogen stimulating Nbs might be a potential therapeutic agent for the treatment of haemophilia A [189]. Nbs used in current study can overcome a reduced TAFI activation problem by stimulating the zymogen activity of TAFI and therefore, can be used as adjuvant therapy next to the factor (VIII and IX) replacement therapy. Moreover, the stimulated zymogen activity might also have a positive effect in other (patho-)physiological processes which requires cleavage of small substrates like blood pressure, rheumatoid arthritis, inflammation and sepsis. In these (patho-)physiological processes TAFIa has a protective effect due to its ability to cleave small molecules like anaphylatoxins (C3a and C5a), bradykinin and thrombin-cleaved-osteopontin [25, 57-61]. Therefore, the importance of the TAFI zymogen stimulation can be evaluated using *in vitro* assays in which a non-activatable TAFI variant is combined with the zymogen stimulatory Nbs or *in vivo* using a mouse arthritis model [190]. However, Nbs used in the current study do not cross-react with mouse and thus cannot be tested in mouse model. Therefore, the generation of zymogen ″stimulating″ Nbs which cross-react with mouse TAFI is required. Furthermore, for therapeutic purposes Nbs should have sufficiently high affinities (nanomolar range) towards the target. However, the Nbs described in the study have lower affinities towards TAFI (micromolar range). A random mutagenesis approach could be used to increase the affinity of the Nbs. Another approach could be mapping of the paratopes on Nbs,which will enable us to mount residues involved in binding to TAFI on a high affinity Nb framework.

Cardiovascular diseases (CVDs)

The world health organization (WHO) defines, CVDs as group of disorders of the blood vessels and the heart including cardiomyopathies, cerebrovascular disease (stroke), congenital heart disease, coronary heart disease (heart attack), deep vein thrombosis and pulmonary embolism, heart failure, hypertension, peripheral arterial disease and rheumatic heart disease. Ischemic stroke and myocardial infarction/heart attack are mainly acute events caused by blockage of artery or vein by a thrombus that prevents blood flow to the brain or heart, respectively, resulting into cell necrosis and loss of organ function. According to the WHO (2008), CVDs are the largest cause of sickness and morbidity worldwide (17.3 million deaths annually, 30% of all the global deaths) with a very high socioeconomic burden. It is estimated that by 2030, 23.6 million people will die annually due to CVDs, mainly by heart disease and stroke [14]. Every year CVDs cause approximately 1.9 million deaths (40% of the total deaths) in the European Union alone and are estimated to cost approximately €196 billion in 2012 (European Cardiovascular Disease Statistics) [191].

Thrombolytic therapy

Currently the available thrombolytic therapy involves the administration of streptokinase (Streptase®) and tissue-type plasminogen activators (t-PA) like variants such as reteplase (Rapilysin®) and tenecteplase (Metalyse®). Streptokinase, a first generation thrombolytic, is of bacterial (*Streptococcus*) origin and therefore is the cheapest fibrinolytic agent available at the moment. Streptokinase exhibits a low fibrin specificity, has a high immunogenicity and is often associated with bleeding complications. Recombinant t-PA (alteplase: Activase® and Actilyse®) is a second generation thrombolytic, which has a high fibrin selectivity but is also associated with a higher risk of bleeding. Tenecteplase and Reteplase are genetically engineered t-PA molecules with an increased fibrin selectivity and increased plasma half-life and belong to third generation thrombolytics. Due to a similar efficacy compared to t-PA and an easy administration, the third generation thrombolytics are preferred over t-PA. However, these third generation t-PA derivatives are still associated with bleeding complications and neurotoxicity. Another limiting factor hampering the success of thrombolytic therapy is the timing of intervention as it is only beneficial when administered within 4.5 hours of onset of symptoms [1, 15, 16, 192, 193]. Therefore the development of more effective and safer thrombolytic therapy is still relevant and researchers are looking for alternative options.

Plasmin is the central enzyme of the fibrinolytic system. Fibrinolysis is initiated when the zymogen plasminogen is converted into active plasmin via the action of plasminogen activators (t-PA and u-PA). Proteolytic cleavage of fibrin by plasmin generates C-terminal lysine residues that bind to plasminogen and t-PA both, thereby stimulating plasminogen activator-mediated plasminogen activation and propagating fibrinolysis. The control of the regulation of fibrinolysis is important since the impairment of fibrinolysis is associated with thrombosis, whereas excessive fibrinolysis is manifested by a bleeding tendency. One of the inhibitors of fibrinolysis is TAFIa. TAFI is a plasma zymogen, which upon proteolytic activation by trypsin-like enzymes (thrombin, the T/TM complex and plasmin) removes C-terminal lysine residues from the partially degraded fibrin blood clot. Thus, TAFIa

abolishes the cofactor function of the lysine residues, impedes t-PA-mediated plasmin formation and attenuates fibrinolysis [7, 10-13]. The TAFIa moiety is thermally unstable at 37°C which contributes to the regulation of the antifibrinolytic properties of TAFIa [48, 49]. The role of TAFIa in fibrinolysis has been studied extensively and TAFI inhibition either by gene deletion or by pharmacological inhibition was reported to enhance endogenous thrombolysis and/or t-PA-induced exogenous thrombolysis as well as to reduce the t-PA requirements in a number of animal models (cfr. Table 1.2, page 15, Chapter 1) [44, 60, 78, 81, 83, 95, 97, 102]. Many clinical studies have correlated TAFI antigen levels with cardiovascular events such as angina pectoris [168], coronary artery disease [169], ischaemic stroke [170], myocardial infarction [73] and venous thrombosis [69]. Therefore, in a broader perspective the rest of the sections of this thesis was designed to identify and characterize biologicals which can later be applied to fine-tune the thrombolytic therapy. Our study also provides evidence for the use of a human TAFI inhibitor as a profibrinolytic agent *in vitro* (**Chapter 3**) and **Chapter 5**). In spite of the increasing list of TAFIa inhibitors, the lack of specificity, the poor bioavailability, the biphasic effect and the toxicity remains a problem with most of the low molecular weight TAFI inhibitors. Alternatively, MA have been established as therapeutic molecules. These MA have a high stability and a high specificity against their target proteins. Although, MA have an extended plasma half life compared to the small molecule inhibitors, intravenous administration, high costs and a long manufacturing time are major limitations of MA over small molecule inhibitors. However, the specificity and the potency of an inhibitor are more critical parameters for clinical applications than the route of application and the cost-effectiveness.

TAFIa exerts its effect by two different mechanisms; (a) by inhibiting plasminogen activation e.g. fibrinolysis and (b) by clearing active inflammatory mediators. Along with fibrin, TAFIa also has substrate specificity towards anaphylatoxins (C3a, C5a), annexin ll, bradykinin, thrombin-cleaved osteopontin and plasmin-cleaved chemerin. Therefore, TAFIa might be involved in a wide range of physiological processes indicating its possible role in angiogenesis, blood pressure, cell migration, inflammation and sepsis [25, 57-61, 194]. Thus, TAFIa may be related to various pathologies either having a protective (e.g. inflammation, abdominal aortic aneurysm, rheumatoid arthritis, unilateral ureter obstruction and wound healing) or a deteriorating (thrombosis, glomerulonephritis/kidney fibrosis, lung fibrosis and pulmonary hypertension) effect [83, 184]. In various recent studies, increased plasma TAFI(a) levels have been found to be associated with different pathological conditions like cancer/multiple myeloma [195-197], diabetic nephropathy [198, 199], early recurrent fetal loss [200], inflammatory bowel disease (crohn′s disease or ulcerates colitis) [201], hypertension [202] and meningococcal infection in children [203] etc. However, the role of TAFI(a) in any of above pathologies, except thrombosis and inflammation is not clearly understood and it is difficult to conclude whether an increase in plasma TAFI(a) is a cause or a result of the pathology. Here, it is also important to note that in all the above mentioned processes, except fibrinolysis in blood vessels (due to localization of TM at the endothelium), plasmin-mediated TAFI activation could be more relevant than T/TM-mediated TAFI activation. Therefore, further studies are required to identify/confirm various human pathological conditions which are clear cut associated with elevated TAFI(a) levels as well as to determine the involvement of different TAFI activators (thrombin, the T/TM complex and plasmin)

associated with those pathologies to develop a potent and selective TAFI(a) inhibitor for the clinical applications.

Evaluation of relative contribution of different TAFI activators in regulation of fibrinolysis

TAFIa is involved in the regulation of fibrinolysis without disturbing the coagulation process. As its name suggests, TAFI can be activated by thrombin alone but its rate of activation is 1250-fold accelerated in presence of TM [38, 41]. From a number of *in vitro* and *in vivo* studies it was deduced that T/TM is the main physiological activator of TAFI [42-44]. However, the localization of TM on the surface of endothelial cells suggests that the T/TM complex would only activate TAFI at the endothelium and often the fibrin-rich blood clot extends away from the endothelium. Therefore, involvement of other TAFI activators cannot be ruled out. Compared to thrombin, plasmin is a 10-fold stronger activator of TAFI and the efficiency of plasmin-mediated activation compared to thrombin is further enhanced in the presence of glycosaminoglycans like heparin. However, the catalytic efficiency of the plasmin-heparin complex is still 10-fold lower compared to the T/TM complex [38, 178]. Studies suggest that a very low concentration (< 2% of the total plasma concentration of TAFI) of TAFIa is required to attenuate fibrinolysis [25, 38]. However, the physiological relevance of plasmin-mediated TAFI activation is a matter of debate. To elucidate the physiological activators of TAFI we have tried to unravel the relative contribution of different TAFI activators (the T/TM complex *vs*. plasmin) in the regulation of fibrinolysis (**Chapter 3 and Chapter 4**). In **Chapter 3** we revealed that along with the T/TM-mediated TAFI activation, the plasmin-mediated TAFI activation also plays an important role in regulation of fibrinolysis. This is supported by *in vitro* studies in which MA selectively inhibiting plasmin-mediated TAFI activation was used (**Chapter 3**) and by *in vitro* studies using TAFI variants that are selectively activatable by plasmin or the T/TM complex (**Chapter 4**). MA that mainly inhibit the plasmin-mediated TAFI activation (e.g. MA-TCK11A9) and the plasmin- and the thrombin-mediated TAFI activation (e.g. MA-TCK22G2) were shown to accelerate fibrinolysis in *in vitro* clot lysis experiments. We confirmed our findings using TAFI variants which are selectively activatable by either the T/TM complex (TAFI-K133A) or by the plasmin (TAFI-P91S). From our data it could be deduced that the plasmin-mediated TAFI activation constitutes to the complete antifibrinolytic effect exerted by TAFI (**Chapter 4**). In an *in vitro* thromboelastometry model using whole blood from human TAFI transgenic mice, MA that inhibit the plasmin-mediated TAFI activation (MA-TCK11A9) and T/TM-mediated TAFI activation (MA-T12D11) revealed similar profibrinolytic effects, whereas an antibody that inhibits plasmin-, thrombin- and T/TM-mediated TAFI activation (MA-TCK27A4) revealed an enhanced fibrinolysis (**Chapter 5**). These data are in accordance with the chandler loop *ex vivo* analysis using whole human blood thrombi [179] and with the *in vivo* evaluation of MA-TCK26D6 in a mouse thromboembolism model [81]. However, a recent unpublished study suggested that MA-TCK11A9 and MA-TCK26D6, in addition to their effect on the plasmin-mediated TAFI activation, can also interfere with the TAFIa activity when large substrates (e.g. fibrin) are involved but not when small substrates like C3a and C5a are involved (Semeraro *et al.*, submitted). This would implicate that the role of plasmin in the activation of TAFI is overestimated in *in vitro* assays using large substrate and in

in vivo experiments. This also unravels a novel mechanism of TAFIa inhibition which can discriminate between large and small substrates. Till date no small molecule inhibitor is known, which can either inhibit TAFI activation or discriminate between TAFIa inhibition for large *vs*. small substrates. Here MA have an advantage over small molecule inhibitors and can be used to develop such type of selective inhibitors against different pathologies.

Prediction of molecular determinants involved in binding of the T/TM complex and plasmin on TAFI

MA are large protein molecules (145 Å x 85 Å x 40 Å), which bind to their target molecules by non-covalent interactions [204]. Studies with MA that interfere with the functional properties of TAFI revealed a selective inhibition of either T/TM- and/or plasmin-mediated TAFI activation in spite of using the same cleavage site (Arg^{92}) suggesting different secondary binding sites for thrombin, the T/TM complex and plasmin in TAFI [88]. Studies on the characterization of different TAFI variants i.e. TAFI-R92A (non-activatable by thrombin, the T/TM complex and plasmin), TAFI-P91S (selectively resistant to T/TM-mediated activation), TAFI-S90P (selectively resistant to plasmin-mediated TAFI activation) and TAFI-R92K (selectively resistant to thrombin-mediated TAFI activation) also confirm that Arg^{92} is the primary binding site for all the TAFI activators (**Chapter 2** and **Chapter 4**) (Fig. 6.1A and B). A recent study revealed that the positively charged amino acid residues Lys^{42-44} of TAFI are the secondary binding site of TM in TAFI (Fig. 6.1A) [175]. In a previous study, it was shown that MA-T12D11 which inhibits exclusively T/TM-mediated TAFI activation recognizes Gly^{66} as the major residue for its binding to TAFI [88]. Lys^{42-44} residues are in close proximity (14-16 Å) of Gly^{66}. Therefore it appears that TM binds to TAFI around Lys^{42-44}. The same study reported that Val^{41} is a major residue in the binding of MA-T94H3 which inhibits T/TM- and plasmin-mediated TAFI activation, whereas involvement of $Ile^{182-183}$ seems to abolish the plasmin-, thrombin- and T/TM-mediated TAFI activation [205].

Vercauteren *et al*. generated the TAFI-K133A variant which appears to be selectively not activatable by plasmin (Fig. 6.1B) [81]. Other mutations around the 12 Å region of Lys^{133} (H126A, S129A, P135A, Y137A generated during epitope mapping of MA-TCK22G2) also exhibited a reduced plasmin-mediated TAFI activation. Therefore, it appears that the region around Lys^{133} of TAFI is involved in plasmin binding. This is also supported by the fact that MA-TCK11A9 and MA-TCK22G2 mainly inhibiting plasmin-mediated TAFI activation bind in close proximity of Lys^{133}. Asn^{87} and Thr^{88} are the major residues involved in the binding of MA-TCK26D6 to TAFI [81]. MA-TCK26D6 also inhibits mainly the plasmin-mediated TAFI activation but it differs from MA-TCK11A9 since it can also inhibit the plasmin-mediated TAFI activation of rat and mouse TAFI. However, to fully unravel the secondary binding sites of plasmin and the T/TM complex, further studies are required. More direct methods like X-ray crystallography or hydrogen-deuterium exchange mass spectroscopy can be used to determine the residues directly involved in binding of plasmin, thrombin and the T/TM complex binding in TAFI.

Human TAFI transgenic mice

The MA and Nbs used in this study were generated towards human TAFI and except for MA-TCK26D6 do not cross-react with mouse TAFI. Therefore, they cannot be evaluated *in vivo* using a mouse model. The amino acid sequence of mouse and rat TAFI share approximately 85% identity with human TAFI, including the conservation of important residues involved in substrate binding, glycosylation and zinc binding. They also share conserved biochemical characteristics with respect to their activatability by the T/TM complex and their antifibrinolytic effect [34-36]. A previous study revealed that addition of human TAFI in mouse and rat plasma increases clot lysis time [35]. Recently, mice expressing human TAFI (hTAFI) have been generated [181]. Therefore, in the last part of the thesis we aimed to set up an *in vivo* thromboembolism mouse model in which the human anti-TAFI MA could be evaluated in order to confirm their inhibitory effect (**Chapter 5**). Surprisingly, hTAFI mice exhibited an ″intermediate″ fibrinolytic phenotype between TAFI KO and WT mice. In addition, a high variability in fibrin deposition in the lungs of these mice prevented us to use hTAFI mice in *in vivo* experiments in which the profibrinolytic properties of anti-human TAFI MA could be evaluated *in vivo*. The ″intermediate″ phenotype might be due to a less efficient activation of human TAFI by the mouse T/TM complex or plasmin and/or due to a less efficient catalytic activity of human TAFIa on mouse fibrin. However, using whole blood from hTAFI mice in rotational thromboelastometry, we were able to develop an alternative *in vitro* model next to the human whole blood thrombi model [179] for the evaluation of the profibrinolytic properties of the anti-human TAFI inhibiting MA.

Adjuvent therapy vs. monotherapy

Studies suggest that TAFI(a) inhibitors can be used as an adjuvant therapy in addition to the currently used thrombolytic therapy against thrombosis. However, at this moment it is not clear whether TAFI(a) inhibitors alone (monotherapy) are able to prevent and/or to treat thrombosis. Various studies reported contradictory results (cfr. Table 1.2, page 15, Chapter 1). Therefore, detailed *in vivo* animal studies as well as clinical studies in human are required to confirm the applicability. A variety of small molecule TAFIa inhibitors have been used in clinical trials. However, their potency, specificity, toxicity and bioavailability are still a concern [103, 192, 193]. Therefore, the enhancement of translational usage of highly potent and highly selective TAFI(a) inhibitors is need of the hour. Along with a rational drug design approach based on best available information on small molecule inhibitors, peptides and MA, extensive studies are required to identify/confirm pathologies associated with increased TAFIa levels as well as to determine the involvement of TAFI activators in different pathologies in order to get a competent TAFI(a) inhibitor into the hospitals.

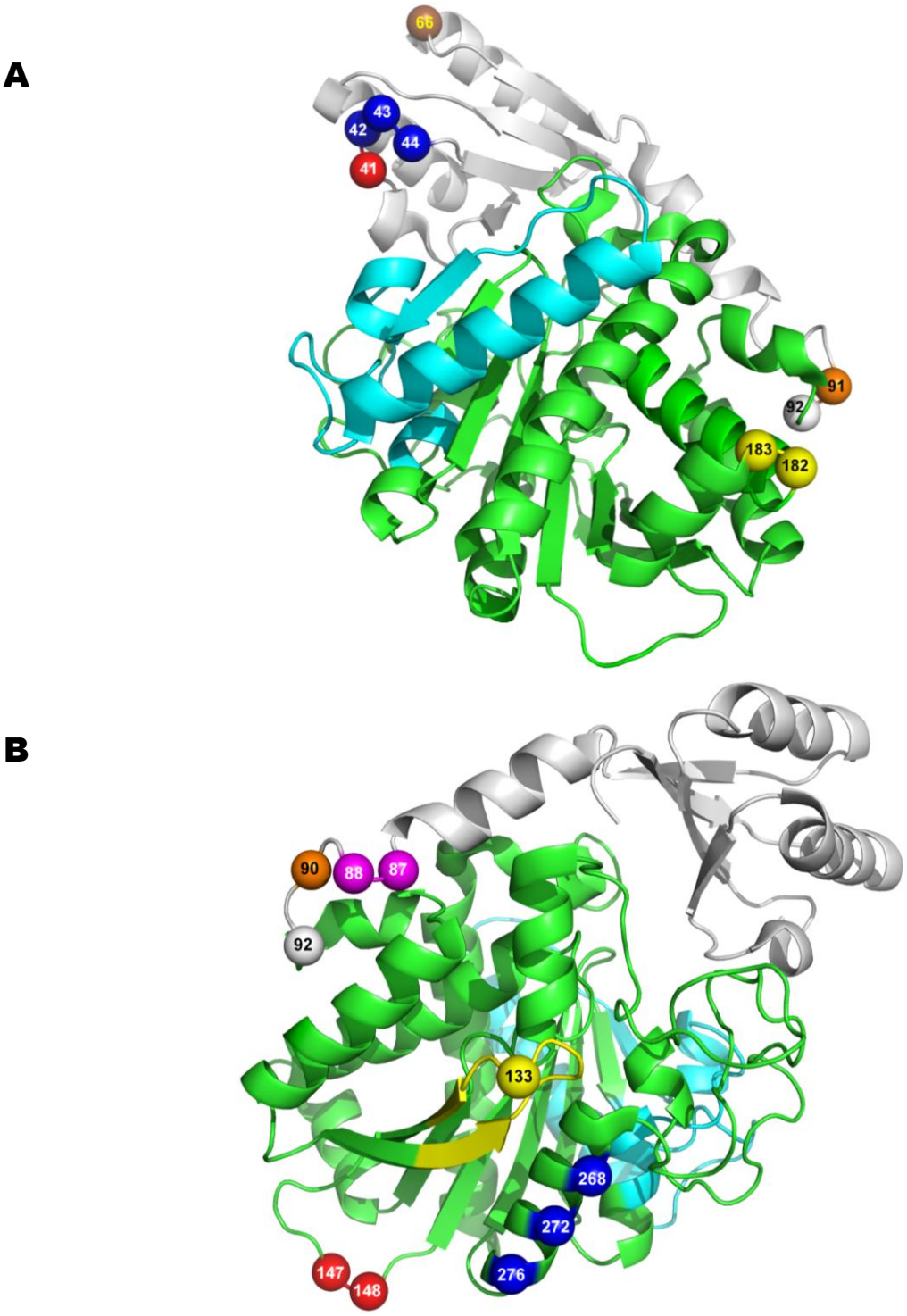

Figure 6.1: Ribbon diagram of TAFI (based on the crystal structure of Marx *et al.* [55]) representing molecular determinants involved in binding of MA as well as the T/TM complex and plasmin on TAFI. The activation peptide, the catalytic domain and the dynamic flap is shown in grey, green and cyan, respectively. Arg^{92} (white sphere) represents primary cleavage site for plasmin, thrombin and the T/TM complex. (A) Epitopes of MA-T94H3 (Val^{41}) and MA-T12D11 (Gly^{66}) are depicted as red and brown spheres, respectively. The residue involved in selective inhibition of TAFI activation by the T/TM complex (Pro^{91}) is represented as orange sphere. Lys^{42-44} (blue spheres) represent the secondary binding site of TM on TAFI. (B) Structure A was rotated around y-axis (180°), z-axis (-35°) and x-axis (-30°) to transform into structure B. Epitopes of MA-TCK11A9 (Lys^{268}, Ser^{272} and Arg^{276}), MA-TCK22G2 (Thr^{147} and Ala^{148}) and MA-TCK26D6 (Asp^{87} and Thr^{88}) are depicted as blue, red and pink spheres, respectively. Residues involved in selective inhibition of plasmin-mediated TAFI activation are represented as yellow (Lys^{133}) and orange (Ser^{90}) spheres. The yellow ribbon depicts the residues present in the 12 Å region of Lys^{133} i.e. His^{126}, Ser^{129}, Pro^{135} and Tyr^{137}.

Future perspective

To conclude, this work unravels that ″stimulated″ zymogen activity exerts an antifibrinolytic effect (**Chapter 2**) and also verifies the use of a human TAFI inhibitor as a profibrinolytic agent *in vitro* (**Chapter 3** and **Chapter 5**). In addition, this study also unravels the relative contribution of the different TAFI activators in the regulation of fibrinolysis (**Chapter 3 and Chapter 4**). However, there is still a long way to go to establish the currently used Nbs and MA as therapeutic molecules (Table 6.1) .

Table 6.1: Lesson learnt and future perspectives.

What have we learnt	What needs to be performed
1. Characterization of TAFI zymogen stimulating Nbs:	
• The ″stimulated(zymogen activity of TAFI exerts an antifibrinolytic effect.	• Could be evaluated in a haemophilia mouse model.
• Similar to the zymogen activity, the (stimulated(zymogen activity might cleave small substrate of TAFI.	• Could be evaluated in in vitro assays • Could be evaluated in an inflammation mouse model.
2. Characterization of anti-human TAFI MA which inhibit TAFI activation by different mechanisms:	
• Irrespective of the mode of inhibition, all MA exhibited a profibrinolytic effect *in vitro*, but cannot be evaluated in hTAFI mice *in vivo*.	• Could be evaluated in non-human primates.
• TAFIa has anti-inflammatory properties and therefore MA may cause inflammation.	• Could be evaluated in *in vitro* assay • Could be evaluated in an inflammation mouse model.

English Summary

Upon proteolytic activation by plasmin, thrombin or the T/TM complex, Thrombin Activatable Fibrinolysis Inhibitor (TAFI) is converted into its active form i.e. TAFIa. TAFI(a) exerts its antifibrinolytic effect by cleaving C-terminal lysine residues from partially degraded fibrin and thereby downregulates the t-PA-mediated activation of plasminogen into plasmin, a major enzyme for fibrin degradation.

Previous studies have demonstrated that TAFI zymogen is an active plasma carboxypeptidase, which exhibits an 18-fold lower catalytic efficiency compared to TAFIa. In this study, we have generated and characterized human-TAFI zymogen stimulating Nbs (Vhh-TAFI-a51 and Vhh-TAFI-i103) and a non-activatable TAFI variant (TAFI-TI-R92A) in order to evaluate the role of the ″stimulated″ zymogen activity of TAFI in the regulation of fibrinolysis (**Chapter 2**). From our analysis, we can conclude that the ″stimulated″ zymogen activity of TAFI can cleave C-terminal lysine residues from larger substrates like partially degraded fibrin, eventually resulting in the prolongation of clot lysis time.

Thromboembolism is one of the major causes of morbidity and mortality in western world and found to be correlated with TAFI(a) antigen levels. Because TAFIa exerts an antifibrinolytic effect, the pharmacological inhibition of TAFI(a) seems to be a promising approach to treat thromboembolism. Therefore, we further evaluated the pharmacological inhibition of human TAFI. We selected three monoclonal antibodies (MA) raised toward human TAFI, which selectively inhibit the activation of human TAFI by plasmin (MA-TCK11A9), by plasmin and thrombin (MA-TCK22G2) and by plasmin, thrombin and the T/TM complex (MA-TCK27A4) and characterized their profibrinolytic properties using *in vitro* assays (**Chapter 3**). These three MA were also used to determine the relative contribution of the different TAFI activators in the regulation of fibrinolysis *in vitro*. We have demonstrated that along with the T/TM complex, plasmin also plays a critical role in the TAFI activation in the regulation of fibrinolysis. This was supported by (a) the profibrinolytic efficacy of MA-TCK11A9, a monoclonal antibody (MA), which mainly inhibits the plasmin-mediated TAFI activation, (b) the ability of MA-TCK11A9 to reduce both TAFIa activity peaks during *in vitro* clot lysis assay and (c) the reduced antifibrinolytic effect of TAFI variants TAFI-P91S and TAFI- K133A which are selectively not activated by the T/TM complex and the plasmin, respectively (**Chapter 3 and Chapter 4**). Therefore, we concluded that plasmin can regulate its own generation through TAFI activation and that the T/TM complex and plasmin both play an important role in TAFI activation in regulation of fibrinolysis.

MA used in present studies cannot be evaluated *in vivo* in mouse model due to their lack of cross-reactivity with mouse TAFI. Recently, mice expressing human TAFI (hTAFI) have been generated. Therefore, we tried to set up an *in vivo* mouse thromboembolism model and an *in vitro* thromboelastometry model using hTAFI transgenic mice to evaluate the effect of anti-human TAFI MA *in vivo* and *in vitro*, respectively (**Chapter 5**). Surprisingly, hTAFI transgenic mice exhibited an ″intermediate″ fibrinolytic phenotype between TAFI KO and WT mice in *in vivo* mouse thromboembolism model. In addition, a high variability in fibrin deposition in lungs of hTAFI transgenic mice prevented us to use these mice for the evaluation of the profibrinolytic properties of anti-human TAFI MA *in vivo*. However, using whole blood from hTAFI mice in rotational thromboelastometry, we were able to develop an alternative *in vitro* model next to the human whole blood thrombi model for the

evaluation of the profibrinolytic properties of the anti-human TAFI MA. The profibrinolytic properties of MA-T12D11, MA-TCK11A9 and MA-TCK27A4 were also evaluated *in vitro* (**Chapter 5**)

To conclude, this study describes the role of the ″stimulated″ zymogen activity of TAFI and elucidates the contribution of the various TAFI activators (plasmin and the T/TM complex) in the regulation of fibrinolysis. We also confirmed the profibrinolytic capacity of the anti-human TAFI MA *in vitro* and *in vitro*. In rotational thromboelastometry using whole blood from hTAFI mice we were able to develop an alternative *in vitro* model in addition to the human whole blood thrombi model for the evaluation of anti-human TAFI MA.

Nederlandse Samenvatting

Thrombin Activatable Fibrinolysis Inhibitor is een zymogeen dat na proteolytische splitsing ter hoogte van Arg^{92} wordt omgezet naar het actief enzyme TAFIa. TAFI(a) splitst eindstandige lysine residues van (partieel gedegradeerd) fibrine. De eindstandige lysines zorgen ervoor dat plasminogeen efficiënter door weefsel-type plasminogeen activator naar plasmine wordt omgezet. TAFIa vermindert de aanmaak van plasmine en vertraagt bijgevolg de fibrinolyse.

Eerdere studies toonden aan dat het zymogeen TAFI ook een carboxypeptidase activiteit bezit. De catalytische efficiëntie van dit proces verloopt echter 18 keer trager in vergelijking met die van TAFIa. In het laboratorium werd een panel van nanobodies tegen TAFI(a) gegeneerd. Karakterisering van de nanobodies toonden aan dat twee van de nanobodies, Vhh-TAFI-a51 en Vhh-TAFI-i103, de carboxypeptidase activiteit van zowel wild-type TAFI als van een inactieve TAFI-TI-R92A variant verhogen (**Chapter 2**). Uit de *in vitro* experimenten bleek dat de “gestimuleerde” zymogeen activiteit van TAFI resulteert in een verlenging van de clot lysis tijd en dus de fibrinolyse vertraagt.

Cardiovasculaire ziekten zijn nog steeds de voornaamste doodsoorzaak in de Westerse wereld. Eerdere studies toonden een correlatie tussen trombose en verhoogde TAFI(a) waarden. TAFI(a) is dus een mogelijke doel eiwit in de ontwikkeling van geneesmiddelen om trombose te voorkomen/behandelen. In deze studie werden drie monoklonale antilichamen geselecteerd die de activatie van humaan TAFI verhinderen. MA-TCK11A9 gaat de activatie van TAFI door plasmine verhinderen. MA-TCK22G2 verhindert de activatie van TAFI door plasmine en trombine terwijl MA-TCK27A4 de activatie van TAFI door plasmine, trombine en trombine/trombomoduline verhindert. Toevoeging van deze drie antilichamen aan plasma resulteerde in een verkorting van de clot lysis. Deze drie antilichamen hebben dus profibrinolytische eigenschappen. Hiermee werd aangetoond dat de activatie van TAFI door zowel plasmine als door het trombine/trombomoduline complex relevant is en bijdraagt tot het antifibrinolytisch effect van TAFI. Deze conclusie werd gestaaft door de volgende bevindingen tijdens *in vitro* clot lysis experimenten: 1) MA-TCK11A9, een monoklonaal antilichaam dat de plasmine gemedieerde TAFI activatie verhindert vertoont een profibrinolytisch effect (**Chapter 3**); 2) MA-TCK11A9 vermindert beide TAFIa activiteitspieken waargenomen tijdens een *in vitro* clot lysis assay (**Chapter 3**); 3) TAFI-P91S en TAFI-K133A, twee TAFI varianten die beide niet/slecht activeerbaar zijn door het trombine/trombomoduline complex maar wel door plasmine, verlengen na toevoeging aan TAFI aan TAFI deficient plasma de clot lysis tijd (**Chapter 4**).

Van de monoklonale antilichamen die opgewekt werden tegen humaan TAFI is er slechts één, namelijk MA-TCK26D6 dat cross-reageert met muis en rat TAFI. Al de andere monoklonalen konden wegens een gebrek aan cross-reactiviteit niet uitgetest worden in een *in vivo* muis model. Om het effect van de verschillende monoklonalen *in vivo* te bestuderen werden transgene muizen die geen muis TAFI maar wel humaan TAFI tot expressie brengen verkregen van Prof. Joost Meijers, AMC Amsterdam. Wanneer echter een tromboembolisme model werd uitgevoerd in deze muizen vertoonden deze een lagere fibrine afzetting in de longen dan de wild-type muizen. De fibrine afzetting was intermediair tussen dat van de TAFI KO muizen en de wild-type muizen. Daarenboven werd er bij deze transgene muizen een grote variatie in fibrine afzetting in de longen na injectie van

tromboplastine waargenomen waardoor het quasi onmogelijk was om deze muizen te gebruiken om het effect van de verschillende monoklonale antilichamen *in vivo* uit te testen. We slaagden er wel in om met vol bloed van deze transgene muizen condities te definiëren waarin het effect van de verschillende monoklonalen in een *in vitro* tromboelastometry model kan getest worden (**chapter 5**).

Als besluit kunnen we stellen dat deze studie heeft bijgedragen om de rol van de "gestimuleerde" zymogeen activiteit van TAFI op te helderen. Deze studie heeft ook de bijdrage van de verschillende TAFI activatoren geillustreerd. Gebruik makend van vol bloed van transgene humane TAFI muizen kon het effect van de anti-humaan TAFI monoklonale antilichamen *in vitro* geconfirmeerd worden.

हिन्दी सारांश

थ्राम्बिन एक्टिवेटेबुल फाइब्रिनोलाइसिस इन्हिबिटर [(टाफी (TAFI)] प्लाज्मिन, थ्रोम्बिन या थ्रोम्बिन/थ्रोम्बोमोडुलिन संकर द्वारा प्रोटिओलिटिक सक्रियण से सक्रिय थ्राम्बिन एक्टिवेटेबुल फाइब्रिनोलाइसिस इन्हिबिटर (टाफ़ी–ए) में परिवर्तित हो जाता है। टाफी–ए आंशिक रूप से विघटित फाइब्रिन के कार्बोक्सि–छोर पर स्थित लाइसिन अवशेषों को फाइब्रिन अणुओं से हटा कर कर टीसू–टाईप प्लाज्मिनोजन एक्टिवेटर उत्प्रेरित प्लाज्मिनोजन के सक्रिय प्लाज्मिन में रूपान्तरण को अधोविनियमित करता है और परिणाम स्वरूप रक्त के जमने से बने थक्के के विघटन (फाइब्रिनोलाइसिस) को रोकता है। प्लाज्मिन फाइब्रिन अपघटन में एक महत्वपूर्ण एंजाइम है।

पूर्व अध्ययनों से प्रदर्शित होता है कि टाफी ज़ाइमोजेन भी एक सक्रिय प्लाज्मा कार्बोक्सिपेप्टिडेज है, जिसकी उत्प्रेरक क्षमता टाफी–ए की उत्प्रेरक क्षमता से अठारह गुना कम है। इस वर्तमान अध्ययन में उत्प्रेरित टाफी जाइमोजेन सक्रियता की भूमिका का फाइब्रिनोलाइसिस के नियमन में मूल्याँकन करने के क्रम में हमने मानवीय–टाफी विशिष्ट जाइमोजन उत्प्रेरक नैनोबॉडियों (nanobodies) और सक्रिय न हो सकने वाले उत्परिवर्ती मानवीय–टाफी प्रतिरूप [टाफी–आर92ए (TAFI-R92A)] को उत्पन्न कर उनका स्वभाव–चित्रण किया (अध्याय २)। हमारे विश्लेषण से यह सत्यापित होता है कि उत्प्रेरित टाफी जाइमोजेन भी आशिंक रूप से विघटित फाइब्रिन जैसे बड़े क्रियाधार (substrate) के कार्बोक्सि–छोर से लाइसिन अवशेषों को हटा कर फाइब्रिनोलाइसिस को नियंत्रित कर सकता है।

थ्राम्बोएम्बोलिज्म (रक्त के थक्का जमने के कारण रक्त–वाहिनियों में उत्पन्न रक्त प्रवाह अवरोध) पाश्चात्य देशों में रुग्णता और मृत्यु के प्रमुख कारणों में से एक है और इसे टाफी/टाफी–ए प्रतिजन के रक्त स्तर से सहसम्बद्ध किया जा सकता है। टाफी–ए की फाइब्रिनोलाइसिस को अधोविनियमित करने की क्षमता के कारण टाफी के सक्रियण/टाफी–ए सक्रियता निषेध को संभावित औषधीय लक्ष्य के रूप में देखा जा रहा है। अतः हमने टाफी सक्रियण के औषधीय/भेषज निषेध (pharmacological inhibition) का अध्ययन किया। फलतः हमने मानवीय–टाफी के विरूद्ध उत्पन्न चार मोनोक्लोनल प्रतिरक्षियों (एन्टीबॉडी), जो चयनात्मक तरीके से क्रमशः थ्रोम्बिन/थ्रोम्बोमोडुलिन संकर [एमए–टी१२डी११ (MA-T12D11)], प्लाज्मिन [एमए–टीसीके११ए९ (MA-TCK11A9)], प्लाज्मिन और थ्रोम्बिन [एमए–टीसीके२२जी२ (MA-TCK22G2)] और प्लाज्मिन, थ्रोम्बिन और थ्रोम्बिन/थ्रोम्बोमोडुलिन संकर [एमए–टीसीके२७ए४ (MA-TCK27A4)] द्वारा मानवीय–टाफी के सक्रियण को रोकती हैं, का अंतः पात्र (इन विट्रो) चरित्र–चित्रण कर इनकी फाइब्रोनोलाइसिस–वर्धन (प्रोफाइब्रिनोलाइटिक) प्रकृति का मूल्यांकन किया (अध्याय ३ और अध्याय ४)। हमने इन मोनोक्लोनल प्रतिरक्षियों का उपयोग फाइब्रिनोलाइसिस के विनिमयन में विभिन्न टाफी सक्रियण कारकों (प्लाज्मिन और थ्रोम्बिन/थ्रोम्बोमोडुलिन) के टाफी सक्रियण में सापेक्षित योगदान के इन–विट्रो अध्ययन में भी किया। अपने परीक्षणों से हमने यह प्रदर्शित किया कि थ्रोम्बिन/थ्रोम्बोमोडुलिन संकर के साथ–साथ प्लाज्मिन भी टाफी सक्रियण द्वारा फाइब्रिनोलाइसिस के विनिमयन में महत्वपूर्ण भूमिका निभाता है। एमए–टीसीके११ए९, जो सिर्फ प्लाज्मिन द्वारा टाफी के सक्रियण को रोकती है; की प्रभावी प्रोफाइब्रिनोलाइटिक क्षमता, एमए–टीसीके११ए९ का इन विट्रो रक्त थक्का लयन परीक्षण में टाफी सक्रियण के दोनों शिखरों को अवनत करने का सामर्थ्य और उत्परिवर्ती टाफी प्रतिरूपों; टाफी–पी९१एस (TAFI-P91S) और टाफी–के१३३ए (TAFI-K133A), जो क्रमशः

चयनात्मक रूप से थ्रोम्बिन/थ्रोम्बोमोडुलिन संकर और प्लाज्मिन द्वारा सक्रिय नहीं किये जा सकते; का क्षीण प्रतिफाइब्रिनोलाइटिक प्रभाव हमारी खोज का समर्थन करता है (अध्याय ३ और अध्याय ४)। अतः हमारे परिणामों के आधार पर हमने यह निष्कर्ष निकाला कि प्लाज्मिन अपने स्व–उत्पादन को टाफी सक्रियण के द्वारा स्वयं नियंत्रित कर सकता है और थ्रोम्बिन/थ्रोम्बोमोडुलिन संकर और प्लाज्मिन दोनों ही टाफी सक्रियण द्वारा फाइब्रिनोलाइसिस के नियंत्रण में महत्वपूर्ण योगदान देते हैं।

इस अध्ययन/अनुसंधान में प्रयुक्त मोनोक्लोनल प्रतिरक्षियों की मूषक–टाफी से अभिक्रियात्मकता के अभाव में इनका मूषक–तन्त्र में इन विवो मूल्यांकन नहीं किया जा सकता। हाल ही में मानवीय–टाफी अभिव्यक्त करने वाले परा–उत्पत्ति मूलक (transgenic) चूहे को उत्पन्न किया गया है। इसलिये हमने मानवीय–टाफी अभिव्यक्त करने वाले चूहे का उपयोग इन विवो मूषक थ्राम्बोएम्बोलिज्म मॉडल और इन–विट्रो थ्रोम्बोएलास्टोमेट्री मॉडल में करके मानवीय–टाफी विशिष्ट मोनोक्लोनल प्रतिरक्षियों के प्रोफाइब्रिनोलाइटिक प्रभाव के मूल्यांकन का प्रयास किया (अध्याय ५)। इन–विवो मूषक थ्रोम्बोएम्बोलिस्म मॉडल में अध्ययन के दौरान मानवीय–टाफी अभिव्यक्त करने वाले चूहे ने आश्चर्यजनक रूप से मूषक–टाफी रहित और मूषक–टाफी सहित चूहे के बीच के फाइब्रिनोलाइटिक लक्षणों (phenotype) को प्रदर्शित किया। इसके अतिरिक्त, मानवीय–टाफी अभिव्यक्त करने वाले चूहे ने इन विवो मूषक थ्राम्बोएम्बोलिज्म मॉडल में चूहे के फेफड़ों में फाइब्रिन निश्रेपण के स्तर में उच्च मात्रा में विविधता प्रदर्शित की। इस कारण हमने मानवीय–टाफी विशिष्ट मोनोक्लोनल प्रतिरक्षियों के प्रोफाइब्रिनोलाइटिक प्रभाव का मूल्यांकन इस मॉडल में नहीं करने का निर्णय लिया। हाँलाकि मानवीय–टाफी अभिव्यक्त करने वाले चूहे के रक्त का उपयोग थ्रोम्बोएलास्टोमेट्री मॉडल में करते हुये हम मानवीय–टाफी विशिष्ट मोनोक्लोनल प्रतिरक्षियों के प्रोफाइब्रिनोलाइटिक प्रभाव के मूल्यांकन के लिये पहले से उपस्थित मानव रक्त थक्का मॉडल के अतिरिक्त एक नये वैकल्पिक इन–विट्रो मॉडल का विकास करने में सफल रहे। इस नये मॉडल का प्रयोग करते हुये हमने मानवीय–टाफी विशिष्ट प्रतिरक्षियों; एमए–टी१२डी११, एमए–टीसीके११ए९ और एमए–टीसीके२७ए४ के प्रोफाइब्रिनोलाइटिक प्रभाव का इन–विट्रो मूल्यांकन भी किया (अध्याय ५)।

सारांश में, यह शोध–प्रबंध फाइब्रिनोलाइसिस विनिमयन में (१) उत्प्रेरित जाइमोजेन सक्रियता की भूमिका और (२) विविध टाफी सक्रियण कारकों (प्लाज्मिन और थ्रोम्बिन/थ्रोम्बोमोडुलिन) के टाफी सक्रियण में सापेक्ष योगदान को स्पष्ट करता है। हमने अपने परीक्षणों द्वारा न सिर्फ मानवीय–टाफी विशिष्ट मोनोक्लोनल प्रतिरक्षियों के प्रोफाइब्रिनोलाइटिक प्रभाव की पुष्टि की बल्कि ऐसे प्रतिरक्षियों के प्रोफाइब्रोनोलाइटिक प्रभाव के मूल्यांकन के लिये इन–विट्रो मॉडल का विकास भी किया।

References

1. Mackman N. Triggers, targets and treatments for thrombosis. *Nature*. 2008; **451**: 914-8.
2. Dahlbäck B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. *J Intern Med*. 2005; **257**: 209-23.
3. Cushman M. Epidemiology and risk factors for venous thrombosis. *Semin Hematol*. 2007; **44**: 62-9.
4. Heit JA. Venous thromboembolism: disease burden, outcomes and risk factors. *J Thromb Haemost*. 2005; **3**: 1611-7.
5. Furie B. Pathogenesis of thrombosis. *Hematology Am Soc Hematol Educ Program*. 2009; **2009**: 255-8.
6. Mishra N. Antithrombotic therapy: current status and future developments. *theHealth* 2012; **3**: 98-108.
7. Adams RL, Bird RJ. Review article: Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants. *Nephrology*. 2009; **14**: 462-70.
8. Sinauridze EI, Panteleev MA, Ataullakhanov FI. Anticoagulant therapy: basic principles, classic approaches and recent developments. *Blood Coagul Fibrinolysis*. 2012; **23**: 482-93.
9. Malý MA, Tomasov P, Hájek P, Blasko P, Hrachovinová I, Salaj P, Veselka J. The role of tissue factor in thrombosis and hemostasis. *Physiol Res*. 2007; **56**: 685-95.
10. Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. *J Thromb Haemost*. 2009; **7**: 4-13.
11. Collen D. The plasminogen (fibrinolytic) system. *Thromb Haemost*. 1999; **82**: 259-70.
12. Booth NA, Bennett B. Fibrinolysis and thrombosis. *Baillieres Clin Haematol*. 1994; **7**: 559-72.
13. Lijnen HR, van Hoef B, Beelen V, Collen D. Characterization of the murine plasma fibrinolytic system. *Eur J Biochem*. 1994; **224**:863-71.
14. World Health Organization (WHO). Cardiovascular diseases (CVDs): Fact sheet N°317. Available: (http://www.who.int/mediacentre/factsheets/fs317/en/index.html) [accessed on 6th June 2013].
15. Kiernan TJ, Gersh BJ. Thrombolysis in acute myocardial infarction: current status. *Med Clin North Am*. 2007; **91**: 617-37.
16. Alias M, Staff I, McCullough LD. Early experience with community implementation of thrombolysis three to 4.5 hours after acute ischemic stroke. *Conn Med*. 2011; **75**: 531-6.
17. Hendriks D, Scharpé S, van Sande M, Lommaert MP. Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N. *J Clin Chem Clin Biochem*. 1989; **27**: 277-85.
18. Campbell W, Okada H. An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits. *Biochem Biophys Res Commun*. 1989; **162**: 933-9.
19. Eaton DL, Malloy BE, Tsai SP, Henzel W, Drayna D. Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. *J Biol Chem*. 1991; **266**: 21833-8.
20. Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *J Biol Chem*. 1995; **270**: 14477-84.
21. Wang W, Hendriks DF, Scharpé SS. Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. *J Biol Chem*. 1994; **269**:15937-44.
22. Vanhoof G, Wauters J, Schatteman K, Hendriks D, Goossens F, Bossuyt P, Scharpé S. The gene for human carboxypeptidase U (CPU)--a proposed novel regulator of plasminogen activation--maps to 13q14.11. *Genomic*s. 1996; **38**: 454-5.
23. Boffa MB, Reid TS, Joo E, Nesheim ME, Koschinsky ML. Characterization of the gene encoding human TAFI (thrombin-activable fibrinolysis inhibitor; plasma procarboxypeptidase B). *Biochemistry*. 1999; **38**: 6547-58.
24. Lin JH, Novakovic D, Rizzo CM, Zagorac B, Garand M, Filipieva A, Koschinsky ML, Boffa MB. The mRNA encoding TAFI is alternatively spliced in different cell types and produces intracellular forms of the protein lacking TAFIa activity. *Thromb Haemost*. 2013; **109**: 1033-44.
25. Boffa MB, Koschinsky ML. Curiouser and curiouser: recent advances in measurement of thrombin-activatable fibrinolysis inhibitor (TAFI) and in understanding its molecular genetics, gene regulation, and biological roles. *Clin Biochem*. 2007; **40**: 431-42.
26. Mosnier LO, Buijtenhuijs P, Marx PF, Meijers JC, Bouma BN. Identification of thrombin activatable fibrinolysis inhibitor (TAFI) in human platelets. *Blood*. 2003; **101**: 4844-6.
27. Lin JH, Garand M, Zagorac B, Schadinger SL, Scipione C, Koschinsky ML, Boffa MB. Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells. *Thromb Haemost*. 2011; **105**: 999-1009.
28. Boffa MB, Wang W, Bajzar L, Nesheim ME. Plasma and recombinant thrombin-activable fibrinolysis inhibitor (TAFI) and activated TAFI compared with respect to glycosylation, thrombin/thrombomodulin-dependent activation, thermal stability, and enzymatic properties. *J Biol Chem*. 1998; **273**: 2127-35.
29. Valnickova Z, Christensen T, Skottrup P, Thøgersen IB, Højrup P, Enghild JJ. Post-translational modifications of human thrombin-activatable fibrinolysis inhibitor (TAFI): evidence for a large shift in the isoelectric point and reduced solubility upon activation. *Biochemistry*. 2006; **45**: 1525-35.
30. Bajzar L, Nesheim ME, Tracy PB. The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. *Blood*. 1996; **88**: 2093-100.
31. Mosnier LO, von dem Borne PA, Meijers JC, Bouma BN. Plasma TAFI levels influence the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. *Thromb Haemost*. 1998; **80**: 829-35.

32. Boffa MB, Maret D, Hamill JD, Bastajian N, Crainich P, Jenny NS, Tang Z, Macy EM, Tracy RP, Franco RF, Nesheim ME, Koschinsky ML. Effect of single nucleotide polymorphisms on expression of the gene encoding thrombin-activatable fibrinolysis inhibitor: a functional analysis. *Blood*. 2008; **111**: 183-9.
33. Gils A, Alessi MC, Brouwers E, Peeters M, Marx P, Leurs J, Bouma B, Hendriks D, Juhan-Vague I, Declerck PJ. Development of a genotype 325-specific proCPU/TAFI ELISA. *Arterioscler Thromb Vasc Biol*. 2003; **23**: 1122-7.
34. Marx PF, Wagenaar GT, Reijerkerk A, Tiekstra MJ, van Rossum AG, Gebbink MF, Meijers JC. Characterization of mouse thrombin-activatable fibrinolysis inhibitor. *Thromb Haemost*. 2000; **83**: 297-303.
35. Hillmayer K, Macovei A, Pauwels D, Compernolle G, Declerck PJ, Gils A. Characterization of rat thrombin-activatable fibrinolysis inhibitor (TAFI)a comparative study assessing the biological equivalence of rat, murine and human TAFI. *J Thromb Haemost*. 2006; **4**: 2470-7.
36. Schatteman KA, Goossens FJ, Scharpé SS, Hendriks DF. Activation of plasma procarboxypeptidase U in different mammalian species points to a conserved pathway of inhibition of fibrinolysis. *Thromb Haemost*. 1999; **82**: 1718-21.
37. Marx PF, Dawson PE, Bouma BN, Meijers JC. Plasmin-mediated activation and inactivation of thrombin-activatable fibrinolysis inhibitor. *Biochemistry*. 2002; **41**: 6688-96.
38. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J Biol Chem*. 1996; **271**: 16603-8.
39. Mao SS, Cooper CM, Wood T, Shafer JA, Gardell SJ. Characterization of plasmin-mediated activation of plasma procarboxypeptidase B. Modulation by glycosaminoglycans. *J Biol Chem*. 1999; **274**: 35046-52.
40. Foley JH, Kim P, Nesheim ME. Thrombin-activable fibrinolysis inhibitor zymogen does not play a significant role in the attenuation of fibrinolysis. *J Biol Chem*. 2008; **283**: 8863-7.
41. Bajzar L, Nesheim M, Morser J, Tracy PB. Both cellular and soluble forms of thrombomodulin inhibit fibrinolysis by potentiating the activation of thrombin-activable fibrinolysis inhibitor. *J Biol Chem*. 1998; **273**: 2792-8.
42. Leurs J, Wissing BM, Nerme V, Schatteman K, Björquist P, Hendriks D. Different mechanisms contribute to the biphasic pattern of carboxypeptidase U (TAFIa) generation during in vitro clot lysis in human plasma. *Thromb Haemost*. 2003; **89**: 264-71.
43. Miah MF, Boffa MB. Functional analysis of mutant variants of thrombin-activatable fibrinolysis inhibitor resistant to activation by thrombin or plasmin. *J Thromb Haemost*. 2009; **7**: 665-72.
44. Binette TM, Taylor FB Jr, Peer G, Bajzar L. Thrombin-thrombomodulin connects coagulation and fibrinolysis: more than an in vitro phenomenon. *Blood*. 2007; **110**: 3168-75.
45. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci*. 1981; **78**: 2249-52.
46. Esmon NL, Owen WG, Esmon CT. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J Biol Chem*. 1982; **257**: 859-64.
47. Foley JH, Cook PF, Nesheim ME. Kinetics of activated thrombin-activatable fibrinolysis inhibitor (TAFIa)-catalyzed cleavage of C-terminal lysine residues of fibrin degradation products and removal of plasminogen-binding sites. *J Biol Chem*. 2011; **286**: 19280-6.
48. Brouwers GJ, Vos HL, Leebeek FW, Bulk S, Schneider M, Boffa M, Koschinsky M, van Tilburg NH, Nesheim ME, Bertina RM, Gomez Garcia EB. A novel, possibly functional, single nucleotide polymorphism in the coding region of the thrombin-activatable fibrinolysis inhibitor (TAFI) gene is also associated with TAFI levels. *Blood*. 2001; **98**: 1992-3.
49. Schneider M, Boffa M, Stewart R, Rahman M, Koschinsky M, Nesheim M. Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *J Biol Chem*. 2002; **277**: 1021-30.
50. Kozian DH, Lorenz M, März W, Cousin E, Mace S, Deleuze JF. Association between the Thr325Ile polymorphism of the thrombin-activatable fibrinolysis inhibitor and stroke in the Ludwigshafen Risk and Cardiovascular Health Study. *Thromb Haemost*. 2010; **103**: 976-83.
51. Boffa MB, Bell R, Stevens WK, Nesheim ME. Roles of thermal instability and proteolytic cleavage in regulation of activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem*. 2000; **275**: 12868-78.
52. Ceresa E, Van de Borne K, Peeters M, Lijnen HR, Declerck PJ, Gils A. Generation of a stable activated thrombin activable fibrinolysis inhibitor variant. *J Biol Chem*. 2006; **281**: 15878-83.
53. Ceresa E, Peeters M, Declerck PJ, Gils A. Announcing a TAFIa mutant with a 180-fold increased half-life and concomitantly a strongly increased antifibrinolytic potential. *J Thromb Haemost*. 2007; **5**: 418-20.
54. Rawlings ND, Morton FR, Barrett AJ. MEROPS: the peptidase database. *Nucleic Acids Res*. 2006; **34**: D270-2
55. Marx PF, Brondijk TH, Plug T, Romijn RA, Hemrika W, Meijers JC, Huizinga EG. Crystal structures of TAFI elucidate the inactivation mechanism of activated TAFI: a novel mechanism for enzyme autoregulation. *Blood*. 2008; **112**: 2803-9.
56. Barbosa Pereira PJ, Segura-Martín S, Oliva B, Ferrer-Orta C, Avilés FX, Coll M, Gomis-Rüth FX, Vendrell J. Human procarboxypeptidase B: three-dimensional structure and implications for thrombin-activatable fibrinolysis inhibitor (TAFI). *J Mol Biol*. 2002; **321**: 537-47.
57. Campbell WD, Lazoura E, Okada N, Okada H. Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. *Microbiol Immunol*. 2002; **46**: 131-4.

58. Myles T, Nishimura T, Yun TH, Nagashima M, Morser J, Patterson AJ, Pearl RG, Leung LL. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. *J Biol Chem*. 2003; **278**: 51059-67.
59. Shinohara T, Sakurada C, Suzuki T, Takeuchi O, Campbell W, Ikeda S, Okada N, Okada H. Pro-carboxypeptidase R cleaves bradykinin following activation. *Int Arch Allergy Immunol*. 1994; **103**: 400-4.
60. te Velde EA, Wagenaar GT, Reijerkerk A, Roose-Girma M, Borel Rinkes IH, Voest EE, Bouma BN, Gebbink MF, Meijers JC. Impaired healing of cutaneous wounds and colonic anastomoses in mice lacking thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost*. 2003; **1**: 2087-96.
61. Asai S, Sato T, Tada T, Miyamoto T, Kimbara N, Motoyama N, Okada H, Okada N. Absence of procarboxypeptidase R induces complement-mediated lethal inflammation in lipopolysaccharide-primed mice. *J Immunol*. 2004; **173**: 4669-74.
62. Willemse JL, Heylen E, Nesheim ME, Hendriks DF. Carboxypeptidase U (TAFIa): a new drug target for fibrinolytic therapy? *J Thromb Haemost*. 2009; **7**: 1962-71.
63. Leurs J, Hendriks D. Carboxypeptidase U (TAFIa): a metallocarboxypeptidase with a distinct role in haemostasis and a possible risk factor for thrombotic disease. *Thromb Haemost*. 2005; **94**: 471-87.
64. Leurs J, Nerme V, Sim Y, Hendriks D. Carboxypeptidase U (TAFIa) prevents lysis from proceeding into the propagation phase through a threshold-dependent mechanism. *J Thromb Haemost*. 2004; **2**: 416-23.
65. Walker JB, Bajzar L. The intrinsic threshold of the fibrinolytic system is modulated by basic carboxypeptidases, but the magnitude of the antifibrinolytic effect of activated thrombin-activable fibrinolysis inhibitor is masked by its instability. *J Biol Chem*. 2004; **279**: 27896-904.
66. Valnickova Z, Thøgersen IB, Potempa J, Enghild JJ. Thrombin-activable fibrinolysis inhibitor (TAFI) zymogen is an active carboxypeptidase. *J Biol Chem*. 2007; **282**: 3066-76.
67. Valnickova Z, Thøgersen IB, Potempa J, Enghild JJ. The intrinsic enzymatic activity of procarboxypeptidase U (TAFI) does not significantly influence the fibrinolytic rate: reply to a rebuttal. *J Thromb Haemost*. 2007; **5**: 1336-7.
68. Willemse JL, Heylen E, Hendriks DF. The intrinsic enzymatic activity of procarboxypeptidase U (TAFI) does not significantly influence the fibrinolysis rate: a rebuttal. *J Thromb Haemost*. 2007; **5**: 1334-6.
69. van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. *Blood*. 2000; **95**: 2855-9.
70. Libourel EJ, Bank I, Meinardi JR, Balje -Volkers CP, Hamulyak K, Middeldorp S, Koopman MM, van Pampus EC, Prins MH, Buller HR, van der Meer J. Co-segregation of thrombophilic disorders in factor V Leiden carriers; the contributions of factor VIII, factor XI, thrombin activatable fibrinolysis inhibitor and lipoprotein(a) to the absolute risk of venous thromboembolism. *Haematologica*. 2002; **87**: 1068-73.
71. Heylen E, Willemse J, Hendriks D. An update on the role of carboxypeptidase U (TAFIa) in fibrinolysis. *Front Biosci*. 2011; **16**: 2427-50.
72. Juhan-Vague I, Morange PE; PRIME Study Group. Very high TAFI antigen levels are associated with a lower risk of hard coronary events: the PRIME Study. *J Thromb Haemost*. 2003; **1**: 2243-4.
73. Juhan-Vague I, Morange PE, Aubert H, Henry M, Aillaud MF, Alessi MC, Samnegard A, Hawe E, Yudkin J, Margaglione M, Di Minno G, Hamsten A, Humphries SE; HIFMECH Study Group. Plasma thrombin-activatable fibrinolysis inhibitor antigen concentration and genotype in relation to myocardial infarction in the north and south of Europe. *Arterioscler Thromb Vasc Biol*. 2002; **22**: 867-73.
74. Meltzer ME, Doggen CJ, de Groot PG, Meijers JC, Rosendaal FR, Lisman T. Low thrombin activatable fibrinolysis inhibitor activity levels are associated with an increased risk of a first myocardial infarction in men. *Haematologica*. 2009; **94**, 811-8.
75. Jood K, Redfors P, Gils A, Blomstrand C, Declerck PJ, Jern C. Convalescent plasma levels of TAFI activation peptide predict death and recurrent vascular events in ischemic stroke survivors. *J Thromb Haemost*. 2012; **10**: 725-7.
76. de Bruijne EL, Gils A, Rijken DC, de Maat MP, Guimarães AH, Poldermans D, Declerck PJ, Leebeek FW. High thrombin activatable fibrinolysis inhibitor levels are associated with an increased risk of premature peripheral arterial disease. *Thromb Res*. 2011; **127**: 254-8.
77. Brouns R, Heylen E, Willemse JL, Sheorajpanday R, De Surgeloose D, Verkerk R, De Deyn PP, Hendriks DF. The decrease in procarboxypeptidase U (TAFI) concentration in acute ischemic stroke correlates with stroke severity, evolution and outcome. *J Thromb Haemost*. 2010; **8**: 75-80.
78. Vercauteren E, Peeters M, Hoylaerts MF, Lijnen HR, Meijers JC, Declerck PJ, Gils A. The hyperfibrinolytic state of mice with combined thrombin-activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor-1 gene deficiency is critically dependent on TAFI deficiency. *J Thromb Haemost*. 2012; **10**: 2555-62.
79. Mao SS, Holahan MA, Bailey C, Wu G, Colussi D, Carroll SS, Cook JJ. Demonstration of enhanced endogenous fibrinolysis in thrombin activatable fibrinolysis inhibitor-deficient mice. *Blood Coagul Fibrinolysis*. 2005; **16**: 407-15.
80. Wang X, Smith PL, Hsu MY, Tamasi JA, Bird E, Schumacher WA. Deficiency in thrombin-activatable fibrinolysis inhibitor (TAFI) protected mice from ferric chloride-induced vena cava thrombosis. *J Thromb Thrombolysis*. 2007; **23**: 41-9.
81. Vercauteren E, Emmerechts J, Peeters M, Hoylaerts MF, Declerck PJ, Gils A. Evaluation of the profibrinolytic properties of an anti-TAFI monoclonal antibody in a mouse thromboembolism model. *Blood*. 2011; **117**: 4615-22.
82. Nagashima M, Yin ZF, Zhao L, White K, Zhu Y, Lasky N, Halks-Miller M, Broze GJ, Jr., Fay WP, Morser J. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. *J Clin Invest*. 2002; **109**: 101-10.

83. Morser J, Gabazza EC, Myles T, Leung LL. What has been learnt from the thrombin-activatable fibrinolysis inhibitor-deficient mouse? *J Thromb Haemost*. 2010; **8**: 868-76.
84. Yoshimoto N, Sasaki T, Sugimoto K, Ishii H, Yamamoto K. Design and characterization of a selenium-containing inhibitor of activated thrombin-activatable fibrinolysis inhibitor (TAFIa), a zinc-containing metalloprotease. *J Med Chem*. 2012; **55**: 7696-705.
85. Schneider M, Nesheim M. Reversible inhibitors of TAFIa can both promote and inhibit fibrinolysis. J Thromb Haemost. 2003; **1**: 147-54.
86. Hendrickx ML, DE Winter A, Buelens K, Compernolle G, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. TAFIa inhibiting nanobodies as profibrinolytic tools and discovery of a new TAFIa conformation. *J Thromb Haemost*. 2011; **9**: 2268-77.
87. Walker JB, Hughes B, James I, Haddock P, Kluft C, Bajzar L. Stabilization versus inhibition of TAFIa by competitive inhibitors in vitro. *J Biol Chem*. 2003; **278**: 8913-21.
88. Gils A, Ceresa E, Macovei AM, Marx PF, Peeters M, Compernolle G, Declerck PJ. Modulation of TAFI function through different pathways--implications for the development of TAFI inhibitors. *J Thromb Haemost*. 2005; **3**: 2745-53.
89. Hillmayer K, Vancraenenbroeck R, De Maeyer M, Compernolle G, Declerck PJ, Gils A. Discovery of novel mechanisms and molecular targets for the inhibition of activated thrombin activatable fibrinolysis inhibitor. *J Thromb Haemost*. 2008; **6**: 1892-9.
90. Buelens K, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. Generation and characterization of inhibitory nanobodies towards thrombin activatable fibrinolysis inhibitor. *J Thromb Haemost*. 2010; **8**: 1302-12.
91. Develter J, Booth NA, Declerck PJ, Gils A. Bispecific targeting of thrombin activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 by a heterodimer diabody. *J Thromb Haemost*. 2008; **6**: 1884-91.
92. Soni H, Sharma A, Bhatt S, Jain MR, Patel PR. Antithrombotic effects due to pharmacological modulation of thrombin-activatable fibrinolysis inhibitor in rats. *Pharmacology*. 2008; **82**: 304-9.
93. Wang X, Smith PL, Hsu MY, Ogletree ML, Schumacher WA. Murine model of ferric chloride-induced vena cava thrombosis: evidence for effect of potato carboxypeptidase inhibitor. *J Thromb Haemost*. 2006; **4**: 403-10.
94. Wang YX, da Cunha V, Vincelette J, Zhao L, Nagashima M, Kawai K, Yuan S, Emayan K, Islam I, Hosoya J, Sullivan ME, Dole WP, Morser J, Buckman BO, Vergona R. A novel inhibitor of activated thrombin activatable fibrinolysis inhibitor (TAFIa) - part II: enhancement of both exogenous and endogenous fibrinolysis in animal models of thrombosis. *Thromb Haemost*. 2007; **97**: 54-61.
95. Nagashima M, Werner M, Wang M, Zhao L, Light DR, Pagila R, Morser J, Verhallen P. An inhibitor of activated thrombin-activatable fibrinolysis inhibitor potentiates tissue-type plasminogen activator-induced thrombolysis in a rabbit jugular vein thrombolysis model. *Thromb Res*. 2000; **98**: 333-42.
96. Islam I, Bryant J, May K, Mohan R, Yuan S, Kent L, Morser J, Zhao L, Vergona R, White K, Adler M, Whitlow M, Buckman BO. 3-Mercaptopropionic acids as efficacious inhibitors of activated thrombin activatable fibrinolysis inhibitor (TAFIa). *Bioorg Med Chem Lett*. 2007; **17**: 1349-54.
97. Klement P, Liao P, Bajzar L. A novel approach to arterial thrombolysis. *Blood*. 1999; **94**: 2735-43.
98. Muto Y, Suzuki K, Iida H, Sakakibara S, Kato E, Itoh F, Kakui N, Ishii H. EF6265, a novel inhibitor of activated thrombin-activatable fibrinolysis inhibitor, protects against sepsis-induced organ dysfunction in rats. *Crit Care Med*. 2009; **37**: 1744-9.
99. Muto Y, Suzuki K, Sato E, Ishii H. Carboxypeptidase B inhibitors reduce tissue factor-induced renal microthrombi in rats. *Eur J Pharmacol*. 2003; **461**: 181-9.
100. Sasaki T, Yoshimoto N, Sugimoto K, Takada K, Murayama N, Yamazaki H, Yamamoto K, Ishii H. Intravenous and oral administrations of DD2 [7-Amino-2-(sulfanylmethyl)heptanoic acid] produce thrombolysis through inhibition of plasma TAFIa in rats with tissue factor-induced microthrombosis. *Thromb Res*. 2012; **130**: e222-8.
101. Hashimoto M, Yamashita T, Oiwa K, Watanabe S, Giddings JC, Yamamoto J. Enhancement of endogenous plasminogen activator-induced thrombolysis by argatroban and APC and its control by TAFI, measured in an arterial thrombolysis model in vivo using rat mesenteric arterioles. *Thromb Haemost*. 2002; **87**: 110-3.
102. Minnema MC, Friederich PW, Levi M, von dem Borne PA, Mosnier LO, Meijers JC, Biemond BJ, Hack CE, Bouma BN, ten Cate H. Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor. *J Clin Invest*. 1998; **101**: 10-4.
103. Vercauteren E, Gils A. Is there any need for a TAFI(a) inhibitor as thrombolytic drug? *Thromb Res*. 2012; **130**: 574-5.
104. Kariolis MS, Kapur S, Cochran JR. Beyond antibodies: using biological principles to guide the development of next-generation protein therapeutics. *Curr Opin Biotechnol*. 2013. (**doi**: 10.1016/j.copbio.2013.03.017).
105. Reichert JM. Next generation and biosimilar monoclonal antibodies: essential considerations towards regulatory acceptance in Europe. February 3-4, 2011, Freiburg, Germany. *MAbs*. 2011; **3**: 223-40.
106. Weisser NE, Hall JC. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. *Biotechnol Adv*. 2009; **27**: 502-20.
107. Lobo ED, Hansen RJ, Balthasar JP. Antibody pharmacokinetics and pharmacodynamics. *J Pharm Sci*. 2004; **93**: 2645-68.
108. Dubel S. *Handbook of Therapeutic Antibodies, Volume I: Technologies.* Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2007.

109. Datta D, Vaidehi N, Xu X, Goddard WA, 3rd. Mechanism for antibody catalysis of the oxidation of water by singlet dioxygen. *Proc Natl Acad Sci USA*. 2002; **99**: 2636-41.
110. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol*. 2005; **23**: 1126-36.
111. Chothia C, Lesk AM. Canonical structures for the hypervariable regions of immunoglobulins. *J Mol Biol*. 1987; **196**: 901-17.
112. Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem*. 2013; **82** :775-97.
113. Galfre G, Milstein C. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol*. 1981; **73**: 3-46.
114. Chiarella P, Fazio VM. Mouse monoclonal antibodies in biological research: strategies for high-throughput production. *Biotechnol Lett*. 2008; **30**: 1303-10.
115. Brekke OH, Sandlie I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov*. 2003; **2**: 52-62.
116. An Z. Monoclonal antibodies - a proven and rapidly expanding therapeutic modality for human diseases. *Protein Cell*. 2010; **1**: 319-30.
117. Carter P. Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer*. 2001; **1**: 118-29.
118. Flego M, Ascione A, Cianfriglia M, Vella S. Clinical development of monoclonal antibody-based drugs in HIV and HCV diseases. *BMC Med*. 2013; **11**: 4.
119. Hoogenboom HR. Selecting and screening recombinant antibody libraries. *Nat Biotechnol*. 2005; **23**: 1105-16.
120. Lonberg N. Human antibodies from transgenic animals. *Nat Biotechnol*. 2005; **23**: 1117-25.
121. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R. Naturally occurring antibodies devoid of light chains. *Nature*. 1993; **363**: 446-8.
122. van der Linden R, de Geus B, Stok W, Bos W, van Wassenaar D, Verrips T, Frenken L. Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama. *J Immunol Methods*. 2000; **240**: 185-95.
123. Blanc MR, Anouassi A, Ahmed Abed M, Tsikis G, Canepa S, Labas V, Belghazi M, Bruneau G. A one-step exclusion-binding procedure for the purification of functional heavy-chain and mammalian-type gamma-globulins from camelid sera. *Biotechnol Appl Biochem*. 2009; **54**: 207-12.
124. Sasso EH, Silverman GJ, Mannik M. Human IgA and IgG F(ab')2 that bind to staphylococcal protein A belong to the VHIII subgroup. *J Immunol*. 1991; **147**: 1877-83.
125. Ewert S, Cambillau C, Conrath K, Plückthun A. Biophysical properties of camelid V(HH) domains compared to those of human V(H)3 domains. *Biochemistry*. 2002; **41**: 3628-36.
126. Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama VH sequences from conventional and heavy chain antibodies. *Mol Immunol*. 1997; **34**: 1121-31.
127. Conrath KE, Lauwereys M, Galleni M, Matagne A, Frère JM, Kinne J, Wyns L, Muyldermans S. Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. *Antimicrob Agents Chemother*. 2001; **45**: 2807-12.
128. Arbabi Ghahroudi M, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett*. 1997; **414**: 521-6.
129. Miller KD, Weaver-Feldhaus J, Gray SA, Siegel RW, Feldhaus MJ. Production, purification, and characterization of human scFv antibodies expressed in Saccharomyces cerevisiae, Pichia pastoris, and Escherichia coli. *Protein Expr Purif*. 2005; **42**: 255-67.
130. Frenken LG, van der Linden RH, Hermans PW, Bos JW, Ruuls RC, de Geus B, Verrips CT. Isolation of antigen specific llama VHH antibody fragments and their high level secretion by Saccharomyces cerevisiae. *J Biotechnol*. 2000; **78**: 11-21.
131. Dumoulin M, Conrath K, Van Meirhaeghe A, Meersman F, Heremans K, Frenken LG, Muyldermans S, Wyns L, Matagne A. Single-domain antibody fragments with high conformational stability. *Protein Sci*. 2002; **11**: 500-15.
132. Dolk E, van der Vaart M, Lutje Hulsik D, Vriend G, de Haard H, Spinelli S, Cambillau C, Frenken L, Verrips T. Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. *Appl Environ Microbiol*. 2005; **71**: 442-50.
133. Ladenson RC, Crimmins DL, Landt Y, Ladenson JH. Isolation and characterization of a thermally stable recombinant anti-caffeine heavy-chain antibody fragment. *Anal Chem*. 2006; **78**: 4501-8.
134. van der Linden RH, Frenken LG, de Geus B, Harmsen MM, Ruuls RC, Stok W, de Ron L, Wilson S, Davis P, Verrips CT. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta*. 1999; **1431**: 37-46.
135. Hagihara Y, Mine S, Uegaki K. Stabilization of an immunoglobulin fold domain by an engineered disulfide bond at the buried hydrophobic region. *J Biol Chem*. 2007; **282**: 36489-95.
136. Saerens D, Conrath K, Govaert J, Muyldermans S. Disulfide bond introduction for general stabilization of immunoglobulin heavy-chain variable domains. *J Mol Biol*. 2008; **377**: 478-88.
137. Hussack G, Hirama T, Ding W, Mackenzie R, Tanha J. Engineered single-domain antibodies with high protease resistance and thermal stability. *PLoS One*. 2011; **6**: e28218.

138. Harmsen MM, van Solt CB, van Zijderveld-van Bemmel AM, Niewold TA, van Zijderveld FG. Selection and optimization of proteolytically stable llama single-domain antibody fragments for oral immunotherapy. *Appl Microbiol Biotechnol*. 2006; **72**: 544-51.
139. De Genst E, Silence K, Decanniere K, Conrath K, Loris R, Kinne J, Muyldermans S, Wyns L. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci USA*. 2006; **103**: 4586-91.
140. Lauwereys M, Arbabi Ghahroudi M, Desmyter A, Kinne J, Hölzer W, De Genst E, Wyns L, Muyldermans S. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J*. 1998; **17**: 3512-20.
141. Decanniere K, Desmyter A, Lauwereys M, Ghahroudi MA, Muyldermans S, Wyns L. A single-domain antibody fragment in complex with RNase A: non-canonical loop structures and nanomolar affinity using two CDR loops. *Structure*. 1999; **7**: 361-70.
142. Coppieters K, Dreier T, Silence K, de Haard H, Lauwereys M, Casteels P, Beirnaert E, Jonckheere H, Van de Wiele C, Staelens L, Hostens J, Revets H, Remaut E, Elewaut D, Rottiers P. Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. *Arthritis Rheum*. 2006; **54**: 1856-66.
143. Baral TN, Magez S, Stijlemans B, Conrath K, Vanhollebeke B, Pays E, Muyldermans S, De Baetselier P. Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. *Nat Med*. 2006; **12**: 580-4.
144. Cortez-Retamozo V, Backmann N, Senter PD, Wernery U, De Baetselier P, Muyldermans S, Revets H. Efficient cancer therapy with a nanobody-based conjugate. *Cancer Res*. 2004; **64**: 2853-7.
145. Vincke C, Loris R, Saerens D, Martinez-Rodriguez S, Muyldermans S, Conrath K. General strategy to humanize a camelid single-domain antibody and identification of a universal humanized nanobody scaffold. *J Biol Chem*. 2009; **284**: 3273-84.
146. Saerens D, Frederix F, Reekmans G, Conrath K, Jans K, Brys L, Huang L, Bosmans E, Maes G, Borghs G, Muyldermans S. Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing. *Anal Chem*. 2005; **77**: 7547-55.
147. Clinical trial search of Ablynx-List results -ClinicalTrials.gov. Available: (http://clinicaltrials.gov/ct2/results?term=ablynx) [accessed on 6th June 2013].
148. Muyldermans S. Single domain camel antibodies: current status. *J Biotechnol*. 2001; **74**: 277-302.
149. Hmila I, Abdallah RB, Saerens D, Benlasfar Z, Conrath K, Ayeb ME, Muyldermans S, Bouhaouala-Zahar B. VHH, bivalent domains and chimeric Heavy chain-only antibodies with high neutralizing efficacy for scorpion toxin AahI'. *Mol Immunol*. 2008; **45**: 3847-56.
150. Marx PF, Plug T, Havik SR, Mörgelin M, Meijers JC. The activation peptide of thrombin-activatable fibrinolysis inhibitor: a role in activity and stability of the enzyme? *J Thromb Haemost*. 2009; **7**: 445-52.
151. Willemse JL, Polla M, Hendriks DF. The intrinsic enzymatic activity of plasma procarboxypeptidase U (TAFI) can interfere with plasma carboxypeptidase N assays. *Anal Biochem*. 2006; **356**: 157-9.
152. Buelens K, Hillmayer K, Compernolle G, Declerck PJ, Gils A. Biochemical importance of glycosylation in thrombin activatable fibrinolysis inhibitor. *Circ Res*. 2008; 102: 295-301.
153. Mishra N, Vercauteren E, Develter J, Bammens R, Declerck PJ, Gils A. Identification and characterisation of monoclonal antibodies that impair the activation of human thrombin activatable fibrinolysis inhibitor through different mechanisms. *Thromb Haemost*. 2011; **106**: 90-101.
154. Ceresa E, Brouwers E, Peeters M, Jern C, Declerck PJ, Gils A. Development of ELISAs measuring the extent of TAFI activation. *Arterioscler Thromb Vasc Biol*. 2006; **26**: 423-8.
155. Valnickova Z, Sanglas L, Arolas JL, Petersen SV, Schar C, Otzen D, Aviles FX, Gomis-Rüth FX, Enghild JJ. Flexibility of the thrombin-activatable fibrinolysis inhibitor pro-domain enables productive binding of protein substrates. *J Biol Chem*. 2010; **285**: 38243-50.
156. Bouma BN, Meijers JC. Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). *J Thromb Haemost*. 2003; **1**: 1566-74.
157. Bouma BN, Marx PF, Mosnier LO, Meijers JC. Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). *Thromb Res*. 2001; **101**: 329-54.
158. Hendriks D, Wang W, Scharpé S, Lommaert MP, van Sande M. Purification and characterization of a new arginine carboxypeptidase in human serum. *Biochim Biophys Acta*. 1990; **1034**: 86-92.
159. Tan AK, Eaton DL. Activation and characterization of procarboxypeptidase B from human plasma. B*iochemistry.* 1995; 3**4:** 5811-6.
160. Marx PF, Hackeng TM, Dawson PE, Griffin JH, Meijers JC, Bouma BN. Inactivation of active thrombin-activable fibrinolysis inhibitor takes place by a process that involves conformational instability rather than proteolytic cleavage. J *Biol Chem.* 2000; **275:** 12410-5.
161. Redlitz A, Tan AK, Eaton DL, Plow EF. Plasma carboxypeptidases as regulators of the plasminogen system. J *Clin Invest.* 1995; 9**6:** 2534-8.
162. Vendrell J, Querol E, Avilés FX. Metallocarboxypeptidases and their protein inhibitors. Structure, function and biomedical properties. *Biochim Biophys Acta*. 2000; **1477**: 284-98.
163. Bouma BN, Mosnier LO, Meijers JC, Griffin JH. Factor XI dependent and independent activation of thrombin activatable fibrinolysis inhibitor (TAFI) in plasma associated with clot formation. *Thromb Haemost*. 1999; **82**: 1703-8.

164. Hillmayer K, Brouwers E, León-Tamariz F, Meijers JC, Marx PF, Declerck PJ, Gils A. Development of sandwich-type ELISAs for the quantification of rat and murine thrombin activatable fibrinolysis inhibitor in plasma. *J Thromb Haemost*. 2008; **6**: 132-8.
165. Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. I*mmunochemistry.* 1978; 1**5:** 429-36.
166. Nakane PK, Kawaoi A. Peroxidase-labeled antibody. A new method of conjugation. J *Histochem Cytochem.* 1974; **22:** 1084-91.
167. Morange PE, Juhan-Vague I, Scarabin PY, Alessi MC, Luc G, Arveiler D, Ferrieres J, Amouyel P, Evans A, Ducimetiere P; PRIME Study group. Association between TAFI antigen and Ala147Thr polymorphism of the TAFI gene and the angina pectoris incidence. The PRIME Study (Prospective Epidemiological Study of MI). *Thromb Haemost*. 2003; **89**: 554-60.
168. Silveira A, Schatteman K, Goossens F, Moor E, Scharpé S, Strömqvist M, Hendriks D, Hamsten A. Plasma procarboxypeptidase U in men with symptomatic coronary artery disease. *Thromb Haemost*. 2000; **84**: 364-8.
169. Montaner J, Ribó M, Monasterio J, Molina CA, Alvarez-Sabín J. Thrombin-activable fibrinolysis inhibitor levels in the acute phase of ischemic stroke. *Stroke*. 2003; **34**: 1038-40.
170. Polla MO, Tottie L, Nordén C, Linschoten M, Müsil D, Trumpp-Kallmeyer S, Aukrust IR, Ringom R, Holm KH, Neset SM, Sandberg M, Thurmond J, Yu P, Hategan G, Anderson H. Design and synthesis of potent, orally active, inhibitors of carboxypeptidase U (TAFIa). *Bioorg Med Chem*. 2004; **12**: 1151-75.
171. Do YH, Gifford-Moore DS, Beight DW, Rathnachalam R, Klimkowski VJ, Warshawsky AM, Lu D. Inhibition of thrombin activatable fibrinolysis inhibitor by cysteine derivatives. *Thromb Res*. 2005; **116**: 265-71.
172. Mutch NJ, Moore NR, Wang E, Booth NA. Thrombus lysis by uPA, scuPA and tPA is regulated by plasma TAFI. J *Thromb Haemost*. 2003; **1**: 2000-7.
173. Guimarães AH, Rijken DC. Thrombin activatable fibrinolysis inhibitor (TAFI) affects fibrinolysis in a plasminogen activator concentration-dependent manner. Study of seven plasminogen activators in an internal clot lysis model. *Thromb Haemost*. 2004; **91**: 473-9.
174. Wu C, Kim PY, Manuel R, Seto M, Whitlow M, Nagashima M, Morser J, Gils A, Declerck P, Nesheim ME. The roles of selected arginine and lysine residues of TAFI (Pro-CPU) in its activation to TAFIa by the thrombin-thrombomodulin complex. *J Biol Chem*. 2009; **284**: 7059-67.
175. Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J *Biol Chem.* 1982; 2**57:** 2912-9.
176. Wang W, Boffa MB, Bajzar L, Walker JB, Nesheim ME. A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. J *Biol Chem.* 1998; **273:** 27176-81.
177. Vercauteren E, Mutch NJ, Declerck PJ, Gils A. Plasmin and the thrombin-thrombomodulin complex both contribute to thrombin-activatable fibrinolysis inhibitor activation in whole blood model thrombi. J *Thromb Haemost.* 2013; **11:** 190-2.
178. Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. *J Thromb Haemost*. 2009; **7**: 4-13.
179. Valls Seron M. Thrombin-Activatable Fibrinolysis Inhibitor and Bacterial Infections. E*xperimental Vascular Medicine.* UvA-DARE, the institutional repository of the University of Amsterdam (UvA): http://dare.uva.nl/document/340526: University of Amsterdam, 2011, 128.
180. Vandevenne E, Van Buggenhout S, Duvetter T, Brouwers E, Declerck PJ, Hendrickx ME, Van Loey A, Gils A. Development and evaluation of monoclonal antibodies as probes to assess the differences between two tomato pectin methylesterase isoenzymes. *J Immunol Methods*. 2009; **349**: 18-27.
181. Vercauteren E, Gils A, Declerck PJ. Thrombin activatable fibrinolysis inhibitor: a putative target to enhance fibrinolysis. *Semin Thromb Hemost*. 2013; **39**: 365-72.
182. Russell, WMS. and Burch, RL. The Principles of Humane Experimental Technique.: Universities Federation for Animal Welfare Wheathampstead, England, UK., 1959 (reprinted in 1992).
183. Shenkman B, Livnat T, Lubetsky A, Tamarin I, Budnik I, Einav Y, Martinowitz U. The in-vitro effect of fibrinogen, factor XIII and thrombin-activatable fibrinolysis inhibitor on clot formation and susceptibility to tissue plasminogen activator-induced fibrinolysis in hemodilution model. B*lood Coagul Fibrinolysis.* 2012; **23:** 370-8.
184. Anand K, Pallares I, Valnickova Z, Christensen T, Vendrell J, Wendt KU, Schreuder HA, Enghild JJ, Avilés FX. The crystal structure of thrombin-activable fibrinolysis inhibitor (TAFI) provides the structural basis for its intrinsic activity and the short half-life of TAFIa. *J Biol Chem*. 2008; **283**: 29416-23.
185. Mosnier LO, Lisman T, van den Berg HM, Nieuwenhuis HK, Meijers JCM, Bouma BN. The defective down regulation of fibrinolysis in haemophilia A can be restored by increasing the TAFI plasma concentration. *Thromb Haemost.* 2001; **86:** 1035-9.
186. Foley JH, Nesheim ME, Rivard GE, Brummel-Ziedins KE. Thrombin activatable fibrinolysis inhibitor activation and bleeding in haemophilia A. H*aemophilia.* 2012; 1**8:** e316-22.
187. Song JJ, Hwang I, Cho KH, Garcia MA, Kim AJ, Wang TH, Lindstrom TM, Lee AT, Nishimura T, Zhao L, Morser J, Nesheim M, Goodman SB, Lee DM, Bridges SL Jr; Consortium for the Longitudinal Evaluation of African Americans with Early Rheumatoid Arthritis (CLEAR) Registry, Gregersen PK, Leung LL, Robinson WH. Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis. J *Clin Invest.* 2011; **121:** 3517-27.

188. Nichols M TN, Scarborough P, Rayner M, Leal J, Luengo-Fernandez R, Gray A. European Cardiovascular Disease Statistics. European Heart Network and European Society of Cardiology, 2012. Available: (http://www.escardio.org/about/Documents/EU-cardiovascular-disease-statistics-2012.pdf)[accessed on 6th June 2013].
189. Kunadian V, Gibson CM. Thrombolytics and myocardial infarction. C*ardiovasc Ther.* 2012; 3**0:** e81-8.
190. Fernandez D, Pallares I, Vendrell J, Aviles FX. Progress in metallocarboxypeptidases and their small molecular weight inhibitors. B*iochimie.* 2010; **92:** 1484-500.
191. Kim PY, Kim PY, Taylor FB, Jr, Nesheim ME. Thrombin-activatable fibrinolysis inhibitor is activated in vivo in a baboon model of Escherichia coli induced sepsis. *J Thromb Thrombolysis.* 2012; 3**3:** 412-5.
192. Kaftan O, Kasapoglu B, Koroglu M, Kosar A, Yalcin SK. Thrombin-activatable fibrinolysis inhibitor in breast cancer patients. *Med Princ Pract*. 2011; **20**: 332-5.
193. Balcik OS, Albayrak M, Uyar ME, Dagdas S, Yokus O, Ceran F, Cipil H, Kosar A, Ozet G. Serum thrombin activatable fibrinolysis inhibitor levels in patients with newly diagnosed multiple myeloma. B*lood Coagul Fibrinolysis.* 2011; **22:** 260-3.
194. Chengwei X, Xiaoli M, Yuan Z, Li P, Shengjiang W, Chao Y, Yunshan W. Plasma thrombin-activatable fibrinolysis inhibitor levels and its Thr325Ile polymorphism in breast cancer. *Blood Coagul Fibrinolysis*. 2013; (**doi**:10.1097/MBC.0b013e3283610381).
195. Xu CW, Wu XB, Ma XL, Wang YS, Zhang BC, Zhao JJ, Wang ZJ, Chen J. Genetic variation in thrombin-activatable fibrinolysis inhibitor is associated with the risk of diabetic nephropathy. *J Endocrinol Invest*. 2012; **35**: 620-4.
196. Małyszko J, Małyszko JS, Hryszko T, Myśliwiec M. Thrombin activatable fibrinolysis inhibitor (TAFI) and markers of endothelial cell injury in dialyzed patients with diabetic nephropathy. *Thromb Haemost*. 2004; **91**: 480-6.
197. Legnani C, Bovara M, Valdrè L, Cosmi B, Caniato A, Palareti G. Risk of early recurrent fetal loss and levels of thrombin-activatable fibrinolysis inhibitor. *Thromb Res*. 2012; **130**: 237-41.
198. Owczarek D, Undas A, Foley JH, Nesheim ME, Jabłonski K, Mach T. Activated thrombin activatable fibrinolysis inhibitor (TAFIa) is associated with inflammatory markers in inflammatory bowel diseases TAFIa level in patients with IBD. *J Crohns Colitis.* 2012; **6**: 13-20.
199. Malyszko J, Tymcio J. Thrombin activatable fibrinolysis inhibitor and other hemostatic parameters in patients with essential arterial hypertension. P*ol Arch Med Wewn.* 2008; **118:** 36-41.
200. Emonts M, de Bruijne EL, Guimarães AH, Declerck PJ, Leebeek FW, de Maat MP, Rijken DC, Hazelzet JA, Gils A. Thrombin-activatable fibrinolysis inhibitor is associated with severity and outcome of severe meningococcal infection in children. *J Thromb Haemost*. 2008; **6**: 268-76.
201. Lynch M, Mosher C, Huff J, Nettikadan S, Johnson J, Henderson E. Functional protein nanoarrays for biomarker profiling. P*roteomics.* 2004; **4:** 1695-702.
202. Marx PF, Havik SR, Bouma BN, Meijers JC. Role of isoleucine residues 182 and 183 in thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost*. 2005; **3**: 1293-300.
203. Sanglas L, Arolas JL, Valnickova Z, Aviles FX, Enghild JJ, Gomis-Rüth FX. Insights into the molecular inactivation mechanism of h*uman activated t*hrombin-**a**ctivatable fibrinolysis inhibitor. *J Thromb Haemost*. 2010; **8**:1056-65

Curriculum Vitae

Scientific curriculum vitae

Personal

Name	Niraj Mishra
Date of birth	April 2*6, 1982*
Place of birth	*Deoria,* Uttar Pr**a**desh, India
Nationality	Indian

Education

1998-2001	Bachelors in Science Ewing Christian College, Allahabad University, India
2003-2005	Master in Biotechnology, Thesis: "*Biochemical and molecular characterizationof Alginolytic Bacterial strain CMC-8*" Promotor: Dr. Sanjeev Chandrakant Ghadi Department of Biotechnology Goa University, Panjim, Goa, India
2009-2013	Ph.D. in Pharmaceutical Sciences Thesis: "Role of TAFI zymogen activity and relative contribution of different TAFI activators in regulation of fibrinolysis" Promotors: Prof. Ann Gils, Prof. Paul Declerck Laboratory for Pharmaceutical Biology KU Leuven, Leuven, Belgium

Publications

PAPERS

Mishra N, Buelens K, Theyskens S, Compernolle G, Gils A, Declerck PJ. Increased zymogen activity of thrombin-activatable fibrinolysis inhibitor prolongs clot lysis. J Thromb Haemost. 2012, 10(6): 1091-9. *(Impact factor: 6.081).*

Mishra N, Vercauteren E, Develter J, Bammens R, Declerck PJ, Gils A. Identification and characterisation of monoclonal antibodies that impair the activation of human thrombin activatable fibrinolysis inhibitor through different mechanisms. Thromb Haemost; 2011, 106(1): 90-101. *(Impact factor: 6.094).*

Mishra N, Declerck PJ, Gils A. The relative contribution of the different TAFI activators regulating fibrinolysis using selectively activatable TAFI variants and monoclonal antibodies selectively inhibiting TAFI activation (manuscript under preparation).

Mishra N, Declerck PJ, Gils A. Evaluation of human-TAFI expressing TAFI-knock out mouse for screening of the profibrinolytic anti-TAFI monoclonal antibodies *(under review in Thrombosis Research).*

ABSTRACTS AT INTERNATIONAL MEETINGS

ORAL PRESENTARION:

Mishra N, Buelens K, Theyskens S, Compernolle G, Gils A, Declerck PJ. Increased Zymogen Activity of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis. The XXIst International Congress on Fibrinolysis and Proteolysis (2012), Brighton, United Kingdom.

Mishra N, Buelens K, Theyskens S, Compernolle G, Gils A, Declerck PJ. Increased Zymogen Activity of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis. BGFW (2012) organized by Belgium Society of Pharmaceutical Sciences, Brussels, Belgium.

Mishra N, Vercauteren E, Develter J, Bammens R, Declerck PJ, Gils A. Identification and characterization of monoclonal antibodies that impair the activation of human TAFI through different mechanisms. The XXth International Congress on Fibrinolysis and Proteolysis (2010), Amsterdam, the Netherlands.

Mishra N, Vercauteren E, Develter J, Bammens R, Declerck PJ, Gils A. Increased Zymogen Activity of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis. BGFW (2011) organized by Belgium Society of Pharmaceutical Sciences, Brussels, Belgium.

POSTER PRESENTARION:

Mishra N, Buelens K, Theyskens S, Compernolle G, Gils A, Declerck PJ. Increased Zymogen Activity of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis. 1st Spring Symposium (2012), organized by Doctoral School, KULeuven, Leuven, Belgium.

Mishra N, Buelens K, Theyskens S, Compernolle G, Gils A, Declerck PJ. Increased Zymogen Activity of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis. Gordon Research Conference on Plasminogen Activation & Extracellular Proteolysis (2012), Ventura, USA.

Mishra N, Buelens K, Theyskens S, Compernolle G, Gils A, Declerck PJ. Increased Zymogen Activity of Thrombin Activatable Fibrinolysis Inhibitor Prolongs Clot Lysis. Gordon Research Seminar on Plasminogen Activation & Extracellular Proteolysis (2012), Ventura, USA.

Mishra N, Vercauteren E, Develter J, Bammens R, Declerck PJ, Gils A. Identification and characterization of monoclonal antibodies that impair the activation of human TAFI through different mechanisms. ULLA Summer School (2011), Parma, Italy.